

University of Alberta

PPAR γ Regulates Airway Epithelial Ion Transport

by

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Abstract

Peroxisome Proliferator activated receptors (PPARs) are widely expressed lipid-sensitive nuclear receptors that are involved in the regulation of metabolism. PPAR γ is a therapeutic target in the treatment of diabetes and insulin resistance that is expressed in the lung epithelium. The function of these receptors in the lung is widely unknown and their effects on lung epithelial ion transport have not been evaluated. Normal human bronchial epithelial (NHBE) cell monolayers were mounted in Ussing chambers and changes in the short-circuit current (SCC) after 48 hours incubation with PPAR γ agonists were measured. PPAR γ agonists reduced SCC in response to stimulation by carbachol and forskolin. The response to apical ATP was increased, while the basolateral ATP response was decreased. CFTR promoter activity was increased while protein expression was unaffected. Bicarbonate secretion was reduced due to decreased expression of NBC1 protein. PPAR γ regulates the transcription of ion transport proteins in the lung epithelium.

For my family, who have always supported me in my academic endeavors.

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List of Symbols, Nomenclature, and Abbreviations

ASL – Airway surface liquid

ATP – Adenosine Triphosphate

Ca^{2+} – Calcium ions

CaCC – Calcium-activated chloride channels

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis Transmembrane Conductance Regulator

Cl^- – Chloride ions

CIC – Voltage-sensitive chloride channels

DNDS – 4,4'-dinitrostilbene-2,2'-disulphonic acid

1-EBIO – 1-ethyle-2-benzimidazolinone

ENaC – Epithelial sodium channel

ΔF508 – Deletion of phenylalanine residue at position 508

HCO_3^- – Bicarbonate ions

Na^+ – Sodium ions

NBC1 – Sodium-bicarbonate cotransporter subtype 1

NECA – 5'-(n-ethylcarboxamido)adenosine

NHBE – Normal human bronchial epithelium

NKCC1 – Sodium-potassium-chloride cotransporter subtype 1

K^+ – Potassium ions

ORCC – Outward-rectifying chloride channels

PPAR – Peroxisome Proliferator Activated Receptor

SCC – Short-Circuit Current

TPAC – tetrapentylammonium chloride

Chapter 1 Introduction

1. Introduction

Many airway diseases are characterized by alterations in the production of the airway surface liquid (ASL) that lines the airways. ASL is a complex fluid composed of proteins, lipids, and salt that is secreted from the epithelia lining the airway. Changes to ASL can cause impaired mucociliary clearance, chronic infection, and, as a result, airway obstruction in affected patients. Epithelial cells regulate ion movement and secrete proteins and, as a consequence, regulate ASL composition. The modulation of ionic secretions in response to stimuli is an important role of airway epithelial cells. Identification of molecules that can alter the normal response of airway epithelial cells to various stimuli may be therapeutically beneficial in a variety of airway diseases. By altering the physiological properties of airway epithelial cells pharmacologically, ASL can be modulated to ameliorate the symptoms of certain airway diseases.

Cystic Fibrosis (CF) is an autosomal recessive genetic disease resulting from mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). The clinical manifestations of CF in the lung are the production of thick, sticky mucus and persistent infection. CFTR functions are described in 1.1.3.1. Mutations in CFTR cause decreased anion secretion in the lung by reduction of CFTR activity, reduced expression of CFTR at the plasma membrane, non-functioning CFTR, truncated CFTR, or lack of expression (2). The most common mutation is $\Delta F508$, a deletion that results in impaired trafficking of CFTR to the plasma membrane. Current therapy focuses on increasing the activity of available CFTR, increasing the amount of CFTR on the plasma membrane, and removal of mucous from the airways. The focus of this thesis is on pharmacological

agents that can potentially alter the expression and/or activity of CFTR and increase anion secretion, potentially relieving symptoms of the disease.

1.1 Airway Epithelial Ion Transport

Ion transport is an essential function of airway epithelial cells. Maintenance of adequate airway surface liquid (ASL) ensures that mucus height allows mucociliary clearance to occur. Ion transport in human bronchial epithelia is polarized: cells express different transport proteins on the apical and basolateral membranes and have tight junctions separating the two membranes. Figure 1.1 shows a scanning electron micrograph of a bovine tracheal wall – the mucus, sol and cilia are visible. The sol layer consists of electrolytes and water secreted by various epithelial cells in the lung.

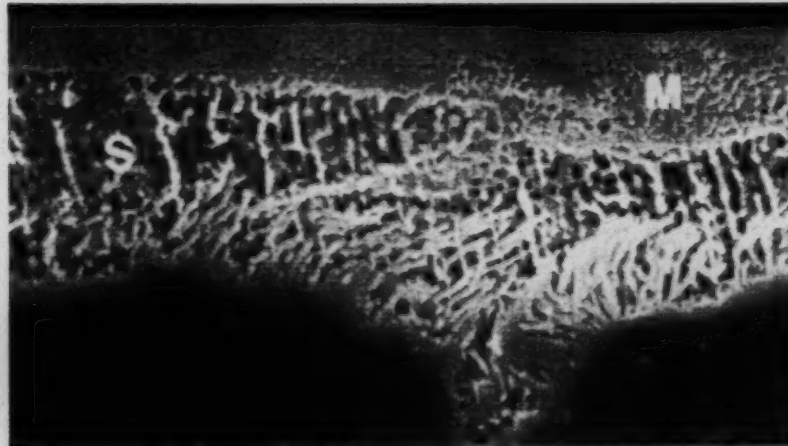


Figure 1.1. Scanning electron micrograph of bovine tracheal wall. M represents the mucous layer, S represents the sol layer, and C represents cilia. Adapted from Wu et al. 1998. (87)

The electrochemical gradient formed by activity of the Na^+/K^+ ATPase in the basolateral membrane drives the diffusion of ions down their respective gradients and into the cell. Sodium diffuses down its electrochemical gradient and into the cell

through, in normal human bronchial epithelial (NHBE) cells, the epithelial sodium channel (ENaC). Potassium diffuses down its chemical gradient and out of the cell through a variety of basolateral potassium channels. Upon activation, chloride travels down its electrical gradient through CFTR and out of the cell. Calcium-activated chloride channels (CaCC) are also expressed in NHBE cells and their function is similar to CFTR. Sodium transport can also be coupled to the transport of other ions into the cell through 2° active transport. Of particular interest to this thesis are NBC1, the basolateral $\text{Na}^+/\text{xHCO}_3^-$ co-transporter, and NKCC1, the bumetanide-sensitive basolateral $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter. NKCC1 is the entry point of Cl^- ions in the basolateral membrane and inhibition results in severe reduction in short-circuit current (SCC). NBC1 is an electrogenic entry point for HCO_3^- ions in NHBE cells. The activity of the different ion transporters is coordinated to allow the electrogenic secretion or absorption of ions to regulate the ASL height and composition.

1.1.1 Chloride Channels

NHBE cells express a variety of chloride channels that coordinate anion secretion. These channels are regulated by several different methods including cAMP, Ca^{2+} , and membrane potential. CFTR, calcium-activated chloride channels (CaCC), and voltage-sensitive chloride channels (CIC) are apically expressed, while outward-rectifying chloride channels (ORCC) channels are expressed on the basolateral membrane. The electrical gradient created by Na^+/K^+ ATPase drives anion efflux from NHBE cells. The selective opening of chloride channels will allow NHBE cells to control movement of

chloride after stimulation with secretagogues. CFTR is responsible for the majority of chloride secretion in NHBE cells.

1.1.1.1 The Cystic Fibrosis Transmembrane Conductance Regulator: CFTR

The Cystic Fibrosis Transmembrane Regulator (CFTR) was identified in 1989 as the genetic defect in patients suffering from Cystic Fibrosis (62). Primary cultures of CF airway epithelia were observed to have reduced apical membrane chloride conductance (9). Single channel studies of CFTR showed that it has a single-channel chloride conductance of ~ 10 pS and was activated by a cAMP-dependent signaling pathway (8). Several different mutations in CFTR can result in a CF phenotype, the most common of which is the $\Delta F508$. CFTR mutations result in degradation of immature CFTR, lack of CFTR synthesis, reduced expression, and impaired channel activity (58). Several different therapies are used to ameliorate lung-specific complications of CF, but effectiveness of these treatments are limited by mucous plugging observed in the diseased lung (17). In cases where CFTR-dependent anion secretion is decreased, orally active pharmacological agents that can increase CFTR expression on the apical membrane of airway epithelial cells could ameliorate airway symptoms of CF.

1.1.1.2 Calcium-activated Chloride Channels: CaCC

Calcium-activated chloride channels (CaCC) have been identified in the human trachea (1). Under normal physiological conditions, these channels are inhibited by CFTR (84). Their importance increases in airway disease, particularly in CF, where chloride conductance through CFTR is absent. UTP has been used clinically to increase

anion secretion through the CaCC channels (7). These channels have also been found to be upregulated in asthma (36). Upregulation of CaCC channel function could increase the effectiveness of airway disease therapies that activate anion secretion via Ca^{2+} .

1.1.1.3 Voltage-sensitive Chloride Channels: ClC

ClC channels are alternate chloride channels in the lung epithelium that may have a role in cystic fibrosis. The expression of ClC-2 subtype has been observed in several human lung cell lines (15, 82), while ClC-5 has been observed in the lungs of adult rats (24). These channels are voltage-regulated and ClC-5 shows outward rectification at positive membrane potentials and may have a functional interaction with ENaC (53, 78). ClC-2 has been shown to increase Cl^- flux in CF cells in response to changes in pH (70). ClC-2G has displayed regulation by cAMP in human lung (73). Increasing the function of these channels may be important to potential CF therapies.

1.1.1.4 Outward-Rectifying Chloride Channels: ORCC

Outward-Rectifying Chloride Channels have been observed in several airway epithelial cell lines including Calu-3, 16HBE14o-, 9HTEo-, and 56FHTE8o- (23, 38, 71). ORCC channels are involved in the selective secretion of bicarbonate during forskolin response in Calu-3 cells (23). These channels are regulated by CFTR and specific mutations in CFTR result in changes in Cl^- flux through ORCC (30, 72). Additionally, cAMP is unable to activate chloride currents through ORCC in CF cell lines (71). These discoveries were possible because ORCC channels display sensitivity to DIDS, while CFTR does not.

1.1.2 ENaC: Epithelial Sodium Channel

ENaC is an amiloride-sensitive sodium channel expressed in the apical membrane of NHBE cells (11). It is proposed to be a tetrameric protein composed of $2\alpha 1\beta 1\gamma$ subunits – although other stoichiometries have been suggested (26). The α -subunit is essential for channel function and is able to conduct sodium when co-expressed with only the β - or γ -subunit. ENaC is highly selective for sodium and has a low single-channel conductance of ~ 4 -5 pS (11). In the lung, ENaC allows sodium to enter epithelial cells down its electrochemical gradient in the electrogenic absorption of sodium from the ASL. The osmotic coupling of water movement to sodium ion movement results in a net absorption of fluid from the ASL (75). Recent studies have shown that chloride can inhibit ENaC function (43, 47). This phenomenon is particularly important to CF, where hyperabsorption of sodium is observed in the lung (9).

1.1.3 Potassium Channels

Several basolateral potassium channels have been characterized in NHBE cells. These channels display differential regulation and functional characteristics. Regardless of how they are regulated, potassium channels are essential for maintenance of the membrane potential that drives both sodium absorption from and anion secretion into the ASL. Potassium efflux through these channels causes hyperpolarization of the cell membrane and increases the electrical force that drives ion movement across the epithelium. Calcium-activated and cAMP-activated potassium channels are of particular importance to anion secretion in normal human bronchial epithelium. Both channels

have been observed in NHBE cells and cAMP and Ca^{2+} -dependent signaling pathways activate anion secretion (50).

1.1.3.1 cAMP Gated K^+ Channels

McCann et al. (50) suggested that two types of potassium channels were responsible for anion secretion observed in airway epithelia. This was based on the observation that charybdotoxin, a calcium-activated potassium channel blocker, did not prevent anion secretion in response to increased cytosolic cAMP caused by isoproterenol. The KvLQT1 channel has been observed in 16HBE14o- and excised nasal polyps (49). This channel is cAMP-sensitive and its function in anion secretion is reduced in CF cells, although its expression is unaffected. These channels can be activated experimentally by forskolin or other compounds that increases cAMP concentrations in the vicinity of the channel. Cell surface receptors that activate cAMP are described in 1.2. These channels are sensitive to several pharmacological agents; in this thesis XE991 will be used to block cAMP-gated K^+ channels (46, 92).

1.1.3.2 Ca^{2+} -Gated K^+ Channels

Calcium-dependent potassium channels, as the name suggests, increase K^+ conductance in response to increased cytosolic Ca^{2+} . Two important Ca^{2+} -gated K^+ channels expressed in NHBE cells: both the Maxi- K^+ (BK) and the Intermediate-Conductance K^+ (IK) channel are present in bronchial epithelium (25, 48). BK channels are inhibited by tetrapentylammonium chloride, while IK channels are inhibited by clotrimazole – allowing the specific contributions of these channels to anion secretion to

be isolated experimentally (12, 20). The opening of either BK or IK channels will result in hyperpolarization of the cell and anion secretion.

1.2 Airway Epithelial Cell Surface Receptors

Airway epithelial cells express a variety of cell surface receptors that can affect ion transport. Stimulation of these receptors activate 2nd messenger systems that alter the ion transport pathway through regulation of the transport proteins discussed in 1.2. Cell surface receptors expressed in airway epithelial cells include ATP receptors, adenosine receptors, and muscarinic receptors. These receptors all activate electrogenic ion secretion across airway epithelia, although different mechanisms are involved.

Activation of these receptors result in the generation of second messengers such as cyclic AMP (cAMP), increased cytosolic Ca^{2+} , or both. P1 and P2 Purine receptors and Muscarinic M_3 receptors are involved in anion secretion in normal human bronchial epithelia.

1.2.1 P2 Purine Receptors: ATP receptors

Several sub-types of ATP receptors have been identified in airway epithelial cell lines. Experiments using the 16HBE14o- cell line, immortalized from primary human epithelial cells, show that the G_q -coupled P2Y subtypes P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁ are expressed in human lung epithelia. The P2Y sub-types 2, 4, and 11 are expressed basolaterally, while 1 and 6 are expressed only in the apical membrane (86). Activation of these receptors results in activation of the Phospholipase C dependant pathway – except P2Y₁₁, which also activates G_s and cAMP signaling. The activation of

this pathway increases cytosolic Ca^{2+} , activating Calcium-sensitive Chloride Channels (CaCC) and CFTR. Stimulation of this receptor is currently clinically relevant as a potential therapy for CF as inhaled UTP or UTP analogues. As CFTR activity is either reduced or absent in CF, the effect of UTP is speculated to occur as a result of increased CaCC activity (7, 54).

P2X receptors are also expressed in human airway epithelial cell lines. These purinergic receptors are calcium-permeable non-selective ligand gated cation channels. The expression of these channels occurs on both apical and basolateral membranes. Subtypes P2X₄ and P2X₅ are expressed in primary airway epithelial cultures and the 16HBE14o- cell line, while subtype P2X₆ has also been observed in 16HBE14o- cells (45, 81). Stimulation of P2X receptors results in a transient rise in intracellular calcium, which should activate anion secretion by activation of calcium-sensitive potassium channels (45, 94).

1.2.2 P1 Purine Receptors: Adenosine Receptors

Anion secretion in airway epithelia is also activated by P1 adenosine-stimulated receptors. mRNA for P1 receptor subtypes A_{2A} and A_{2B} has been detected in NHBE cells (80). Both A₂ receptors act through activation of the cAMP dependent signaling pathway, while A_{2B} may also activate the PLC signaling pathway (37) (10). A_{2B} receptors have been functionally demonstrated on the apical membrane of 16HBE14o- and both membranes of Calu-3 cells, while A_{2A} has been demonstrated on the basolateral membrane (10, 34, 80). 5'-(N-ethylcarboxamido) adenosine (NECA) is used experimentally as an adenosine analogue to activate both A_{2A} and A_{2B} receptors.

1.2.3 Muscarinic Receptors

Muscarinic receptors are expressed in human bronchial epithelium and smooth muscle cells. The M_3 subtype is expressed in the basolateral membrane of NHBE cells (3). Muscarinic receptors on airway epithelial cells respond to acetylcholine (ACh) release by parasympathetic neurons to increase airway ion secretion (91). Activation of the M_3 receptors activates the PLC signaling pathway and activates ion secretion. Carbachol (carbamylcholine) is used experimentally as a muscarinic agonist to stimulate anion secretion in airway cells.

1.3 PPAR γ Physiology

The physiological role of PPAR γ is vague. PPAR γ agonists have been shown to increase adipocyte differentiation, increase transcription of proteins involved in glucose sensing, and are used clinically in the treatment of Type II diabetes mellitus (28, 33, 42). In comparison with the known effects of PPAR α , which increases catabolism of fatty acids, PPAR γ appears to be involved in the detection and storage of excess energy. However, the expression of PPAR γ in tissues that are not involved in energy regulation suggests that this receptor may have a larger physiological role than increasing energy storage. Figure 1.2 shows the mechanism of action of endogenous and exogenous PPAR agonists.

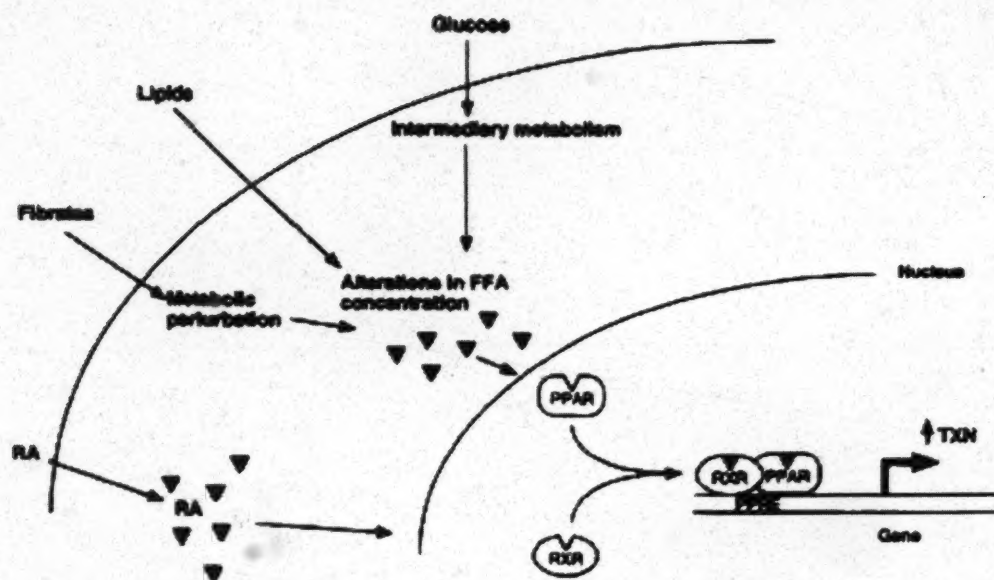


Figure 1.2. Mechanism of PPAR activation of transcription. Aside from alterations in lipid metabolism, pharmacological activators bind directly to PPARs to activate or repress transcription of target genes. Abbreviations: RA – Retinoic Acid; TXN – Transcription; FFA – Free Fatty Acid. Adapted from Schoonjans et al., 1996. (69)

The binding pocket of PPARs is much larger than expected, when compared to other nuclear receptors, suggesting that these receptors are capable of binding a variety of lipophilic molecules (89). The widespread expression and promiscuous binding properties of PPARs suggest that their physiological role involves more than metabolic regulation. It is possible that PPARs in general act as an “energy switch,” detecting cellular energy levels and altering transcription of target genes in response. This hypothesis is supported by observations that PPAR α activation produces similar effects physiologically to caloric restriction (14). The energy switch hypothesis is imperfect in that PPARs have been shown to have other physiological roles, such as modulating inflammation. Several prostaglandins and metabolites have been shown to act as high affinity agonists for PPAR γ . It should be noted that the physiological levels of some

prostaglandin-metabolites do not reach agonist concentrations under normal physiological conditions (5, 59). Treatment with PPAR γ agonists have been shown to reduce acute inflammation in rats and decrease cytokine release from human bronchial epithelial cells (18, 83). The anti-inflammatory effects of PPAR γ also extend into the cardiovascular system. Improvements in cardiovascular health markers have been observed in patients in double-blind studies with PPAR γ agonists (29). PPAR γ agonists also show promise in the treatment of inflammatory lung diseases and acute lung injury (77). The role of PPAR γ in lung ion transport has not been examined experimentally, although effects on ion transport in the rat kidney have been evaluated. In kidneys of rats fed rosiglitazone, the expression of NKCC2, NHE3, aquaporins, and the α -subunit of Na⁺/K⁺ ATPase were increased after 3 days (76). While this does not equate to alterations in lung ion transport, it suggest that PPAR γ may regulate human ion transport proteins.

Observation of altered physiology in subjects with mutations in PPAR γ also provides insight into its physiological roles. Surprisingly, both loss-of-function and mutations that result in constitutively active PPAR γ have nearly identical phenotypes. Dominant negative mutations in PPAR γ isoform 2 (PPAR γ 2) result in Type II diabetes mellitus, severe insulin resistance, and hypertension – a potentially counterintuitive finding based on the proposed physiological role of PPAR γ (4). Hyperactive PPAR γ 2 also results in Type II diabetes mellitus, insulin resistance, and hypertension (63). However, subjects with hyperactive PPAR γ are clinically obese while the dominant negative mutant subjects had normal body mass index (BMI). Considering the ability of PPAR γ to increase insulin sensitivity, diabetes mellitus secondary to reduced insulin

sensitivity appears logical (52, 90). Based on the normal BMI observed in loss-of-function mutations, Type II diabetes in these cases appears to have developed secondary to impaired insulin sensitivity. Unfortunately, there are no studies involving PPAR γ mutants and lung function, which is the primary focus of this thesis.

1.4 Hypothesis and Specific Aims

As transcriptional regulators, PPAR γ agonists represent a promising therapy for airway disease. Clinical studies of pioglitazone-therapy revealed that patients receiving the PPAR γ agonist pioglitazone had an increased incidence of upper respiratory tract infections – suggesting that PPAR γ agonists have regulatory roles in the immune response of the lung (33). As mucociliary clearance is involved in innate immunity, this finding may be a result of impaired mucous clearance. This thesis focuses on ion transport across normal human bronchial epithelium, one aspect responsible for the maintenance of adequate mucociliary clearance. PPAR γ acts through regulation of transcription, it is probable that many different proteins involved in ion transport are affected by PPAR γ activation.

The main objective of this thesis is to evaluate possible changes in lung epithelial ion transport. These changes may be the result of changes in expression of transport proteins, alterations in cellular signaling pathways, and changes in the intracellular handling of transport proteins. The benefits of this research include: 1) potential for discovery of a novel therapy for airway disease, 2) elucidation of PPAR γ regulated transport proteins, and 3) discovery of potential contraindications for PPAR γ agonists for the treatment of dyslipidemia or Type II diabetes mellitus.

Summarized, the specific aims of this thesis are:

1. To evaluate the effects of PPAR γ stimulation on ion transport in normal human bronchial epithelia
2. To explore molecular mechanisms of PPAR γ induced changes in ion transport in normal human bronchial epithelia
3. To investigate the potential of PPAR γ agonists as therapy for airway disease

Chapter 2 Materials and Methods

2.1 Materials

Amiloride, carbachol, thapsigargin, furosemide, 5'-(n-ethylcarboxamido)adenosine (NECA), tolbutamide, clotrimazole, tetrapentylammonium chloride (TPAC), 1-ethyl-2-benzimidazolinone (1-EBIO), 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS), ATP, pioglitazone, FMOC-L-leucine, GW1929, and ouabain were obtained from Sigma-Aldrich Canada (Oakville, Ont). Barium chloride was obtained from Fischer (Pittsburg, PA) and forskolin was purchased from LC Laboratories (Woburn, MD). Xe991, a selective inhibitor of cAMP activated potassium channels, was a generous gift of Dr. B.S. Brown (DuPont, Wilmington, DE). NHBE cells and assay medium were obtained from MatTek (Ashland, MD). 7.5% Polyacrylamide gels, protein assay reagent, and protein markers were obtained from BioRad Laboratories (Hercules, CA). Monoclonal CFTR antibodies were obtained from RD Labs (Washington, MO) and polyclonal β -actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-hNBC1 antibodies were a generous gift of Dr. Joseph R. Casey (University of Alberta, Alta). LUX® quantitative-PCR primers and kit were obtained from Invitrogen (Burlington, Ont). Dual-Luciferase Assay kit and pRL-TK plasmid were obtained from Promega (Madison, WI). CFTR reporter plasmids containing 3.9kb and 0.7kb CFTR promoters were a generous gift of Dr. Mitchell R. Drumm (Case Western Reserve University, OH). Effectene® transfection reagent was obtained from Qiagen (Mississauga, Ont).

2.2 Cell Culture

NHBE cells are the preferred cells for use in this study. These cells are primary cultures of very low passage number ($p \leq 3$). These cells do not retain their normal characteristics after multiple passages, so it was necessary to obtain new cultures on a regular basis. All cultures will be obtained from donors of similar age and genetic background. These cells form confluent, differentiated monolayers that retain the characteristics of *in vivo* airway epithelial cells when grown on an air-liquid interface. NHBE cells are able to form tight junctions – a necessary requirement for use in Ussing chamber experiments. Additionally, NHBE cells have been shown to express several proteins of interest to this study such as PPAR γ , CFTR, and eNaC in cell culture (40). As a result, these cells are an optimal model for examining ion transport in intact human airway epithelium. These cells were obtained from MatTek (Ashland, MD), already passaged onto Costar[®] 12mm Transwell[™] 0.4 μ m pore polyester membranes (Corning, NY). Cells were incubated on an air-liquid interface at 37°C in a 5% CO₂ – 95% air humidified incubator, with medium changed every 2-3 days until drug treatment. Cells were treated with drug dissolved in the media for 48 hours with the assay medium changed daily to ensure fairly constant concentrations of drug. To ensure consistency of treatment, control cell media was also changed daily during the 48-hour treatment period.

A549 cells are a human lung adenoma cell line commonly used in reporter gene studies. These cells do not normally express CFTR, but contain all cellular machinery required to do so. These cells maintain their characteristics over a large number of passages and are ideal for reporter gene assays. A549 cells were grown in Dulbecco's

Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and containing antibiotics. Cells were grown at 37°C in a humidified 5% CO₂ – 95% air incubator. Medium was replaced every 2-3 days with fresh medium. When cells approached 80% confluence, cells were subcultured using 0.05% W/V trypsin – 0.02% W/V ethylene-diamine tetra-acetic acid (EDTA) at 37°C for 10-15 minutes. Cells were then plated onto 24 well plates for transfection and grown until 60% confluent.

2.3 Ussing Chamber Experiments

2.3.1 Intact Cell Measurements

2.3.1.1 Experiments in Normal Krebs-Heinslett Solutions

To assess the effects of PPAR γ stimulation on ion transport, Ussing chambers were employed as a method of measuring net ion flow across the epithelium. NHBE cells were bathed in Krebs-Heinslett Solution (KHS) bubbled with 5% CO₂ balance O₂ in both the apical and basolateral chambers. Normal KHS is composed of NaCl 116 mM, KCl 4.7 mM, CaCl₂ 2.5 mM, MgCl₂ 1.2 mM, NaHCO₃ 24.8 mM, KH₂PO₄ 1.2 mM, and glucose 11.1 mM. Short-circuit current (SCC) was recorded with PowerLab (ADInstruments, CO) and downloaded to a computer. Baseline characteristics of each membrane were recorded, followed by treatment with amiloride apically to block sodium absorption. After each treatment with each ion transport modulator, the SCC was allowed a 10-minute period to reach a steady-state. Figure 2.1 shows a typical experimental recording using carbachol to activate an SCC response. After initial experiments, membranes and chambers were washed with KHS and a second set of

experiments were performed. Table 2.1 shows the different ion transport modulators used, their working concentrations, and their actions in NHBE cells.

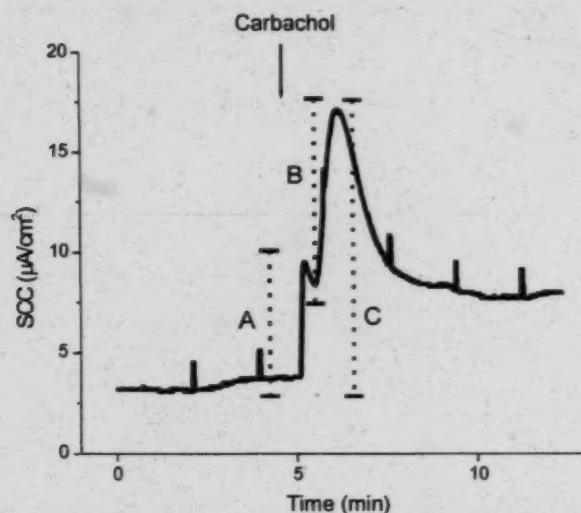


Figure 2.1. A typical NHBE monolayer SCC response to carbachol showing a biphasic response. Section A represents the 1st phase response, Section B represents the 2nd phase response, and Section C represents the peak SCC response due to carbachol. These terms will be used to describe the SCC response of NHBE monolayers to different ion transport modulators.

Modulator	Working Concentration	Function in Ussing chamber experiments
Amiloride	10 μ M	Inhibit ENaC and sodium absorption
ATP	10 μ M	Activate P2 purinergic receptors and induce anion secretion
Barium Chloride	5 mM	Non-specific inhibition of potassium channels
Carbachol	100 μ M	Activate M3 receptors and induce anion secretion
Clotrimazole	50 μ M	Inhibit of the IK potassium channel
DNDS	2 mM	Inhibit basolateral sodium bicarbonate cotransporter
1-EBIO	600 μ M	Activate basolateral potassium channels and induce anion secretion
Forskolin	10 μ M	Increase cytosolic cAMP and induce anion secretion
Furosemide	1 mM	Inhibit the sodium/potassium/chloride cotransporter
NECA	10 μ M	Activate P1 purinergic receptors and induce anion secretion
Ouabain	100 μ M	Inhibit the sodium/potassium ATPase pump
Tetrapentylammonium chloride		Inhibition of the BK potassium channel
Thapsigargin	10 μ M	Inhibit SERCA and induce anion secretion due to increased cytosolic calcium
Tolbutamide	100 μ M	Inhibit ATP-sensitive potassium channels
XE991	30 μ M	Inhibit cAMP-dependent potassium channels

Table 2.1 Anion secretion modulators and their use in Ussing chamber experiments

2.3.1.2 Experiments in Low-Chloride KHS

To isolate the contribution of chloride ion transport to the net current observed in normal KHS, a modified KHS was employed. The protocol used was the same as in 2.3.1.1 with the normal KHS solutions. Gluconate was used as a replacement ion for chloride in the modified KHS. The make-up of low-chloride KHS (lcKHS) is sodium gluconate 116 mM, potassium gluconate 4.7 mM, calcium gluconate 5.0 mM, MgCl_2 1.2 mM, NaHCO_3 24.8 mM, KH_2PO_4 1.2 mM, and glucose 11.1 mM. Since gluconate is an impermeant ion, the net current measured will be due to movement of HCO_3^- across the membrane.

2.3.1.3 Experiments in Bicarbonate-free KHS

To isolate the contribution of bicarbonate transport to the current measured in normal KHS, a second modified KHS was employed. Again, the measurement of net ion transport followed the protocol in 2.3.1.1. Bicarbonate was removed from solution and replaced with HEPES buffered to pH 7.4. The composition of bicarbonate-free KHS (bfKHS) is NaCl 135.2 mM, KCl 4.7 mM, CaCl_2 2.5 mM, MgCl_2 1.2 mM, KH_2PO_4 1.2 mM, HEPES 10.0 mM, and glucose 11.1 mM. The solution was bubbled with 100% O_2 to ensure that the bfKHS remained bicarbonate-free. Anion secretion observed with this method can be attributed to electrogenic chloride flux.

2.3.2 Apical Membrane Studies

NHBE cells form polarized monolayers and therefore both apical and basolateral factors may contribute to any changes in anion secretion we observe during the studies outlined in 2.3.1. As a result, it will be necessary to isolate the apical and basolateral membranes to determine what factors may be effected. Cuthbert (16) described a technique for isolating the apical membrane of epithelial cell patches using a depolarizing high-potassium and low-chloride solution. This solution depolarizes the basolateral membrane and the low-chloride content results in an apical to basolateral chloride gradient. Chambers will be balanced with normal KHS before replacement of the basolateral solution with the membrane-depolarizing KHS (bdKHS). The composition of bdKHS is K gluconate 116 mM, KCl 4.7 mM, Ca gluconate 5.0 mM, MgCl_2 1.2 mM, KHCO_3 24.8 mM, KH_2PO_4 1.2 mM, and glucose 11.1 mM. The apical membrane of will be treated with amiloride to block sodium conductance and BaCl_2 to block potassium conductance. After a baseline is reached, the membranes were treated with forskolin bilaterally to activate apical anion channels and, due to the apical to basolateral gradient, chloride absorption. Changes in apical anion conductance will be observed as negative current.

2.3.3 Basolateral Membrane Studies

2.3.3.1 Bicarbonate Transporter Studies

To determine whether changes in basolateral membrane ion transport contribute to changes in net ion flux, another modified Ussing chamber technique was used. The contributions of total potassium channel activity were already examined in the intact cell

preparations, this technique exams the effect of PPAR γ activation on NBC1 and the basal open state of potassium channels. A modified voltage-clamp technique was employed using the Ussing chamber. Sodium concentrations in both the apical and basolateral solution were modified to isolate the current attributed to NBC1. The apical solution was composed of sodium gluconate 10 mM, HEPES 110 mM, CaCl₂ 5 mM, MgCl₂ 1.2 mM, NaHCO₃ 25 mM, KH₂PO₄ 1.2 mM, and glucose 11.1 mM. The basolateral solution was composed of sodium gluconate 50 mM, N-methyl-D-glucamine (NMDG) 70 mM, CaCl₂ 5 mM, MgCl₂ 1.2 mM, NaHCO₃ 25 mM, KH₂PO₄ 1.2 mM, and glucose 11.1 mM. Cells were clamped at 0 mV and treated apically with nystatin to permeabilize the apical membrane similar to the method used by Gross et al (31). After the SCC stabilized, cells were subjected to a progression of potentials from +50 mV to -50 mV in 10 mV steps. At each voltage step, the cells were allowed to reach a steady-state before being subjected to the next potential. Then the potential was again clamped at 0 mV and the cells were treated with DNDS 2 mM basolaterally to inhibit NBC1. After a baseline was reached, cells were again subjected to the voltage clamp from +50 to -50 mV. The values recorded for each potential before and after DNDS treatment were subtracted to find the contribution of NBC1 to the whole cell current.

2.3.3.2 Potassium Channel Studies

One hypothesis to explain the lowered SCC response to forskolin and carbachol stimulation was that potassium channels in PPAR γ agonist treated cells had higher baseline potassium channel activity. To examine the effect of PPAR γ stimulation on the basal state of basolateral potassium channels, basolateral membrane only Ussing chamber

technique was employed. Sodium gluconate KHS (sgKHS) was used as the basolateral solution and bdKHS was employed as the apical solution. sgKHS is composed of sodium gluconate 116 mM, KCl 4.7 mM, calcium gluconate 5.0 mM, MgCl_2 1.2 mM, KHCO_3 24.8 mM, KH_2PO_4 1.2 mM, and glucose 11.1 mM. The use of these solutions creates an apical to basolateral potassium gradient that allows the measurement of potassium movement across the basolateral membrane. The apical cell membrane is not permeable to potassium and will be permeabilized using nystatin (180 $\mu\text{g/ml}$). Once the SCC reaches a steady-state, BaCl_2 will be added to the basolateral solution to inhibit the potassium channels, giving an indication of the basal potassium current.

2.4 Quantitative PCR

Real-time reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) was used to measure changes in total cellular mRNA concentration of proteins of interest. Intron-spanning LUX[®] (Light Upon eXtension) primers were used designed using the Invitrogen primer design website. This technique allows quantification of the changes expression at the mRNA level. The abundance of mRNA for CFTR, NBC1, NKCC1, and the molecular standard β -actin were determined using RT-qPCR. The CFTR, NKCC1, and NBC1 primers were conjugated to the fluorescent dye FAM, while the β -actin primers were conjugated to the fluorescent dye JOE. SuperScript PCR kits (Invitrogen) were used in the PCR reaction. The master reaction mix included MgSO_4 5 mM, 2XReaction Mix 1X, β -actin JOE-labeled primer 100 nM, β -actin unlabeled primer 100 nM, FAM labeled primer 200 nM, unlabeled primer 200 nM, ROX Reference Dye 1X, and SuperScript III RT/Platinum Taq Mix 0.4 μl /20 μl reaction. Cytosolic RNA

from NHBE monolayers used in Ussing chamber experiments was quantified with UV absorbance and diluted to a stock concentration of 0.2 $\mu\text{g}/\mu\text{l}$. For the PCR reactions, three different RNA quantities were used: 20, 30, and 40 ng. This acts as a control for both RNA loading and the effectiveness of the PCR reaction as the cycle numbers observed should decrease with increasing RNA concentration. If RNA loading was consistent, a linear relationship should be observed in count number. This linear relationship will allow the normalization of CFTR or NBC1 count number using β -actin. In addition, a no template control (NTC) that did not have RNA added was used to ensure that the master mix did not become contaminated with external sources of RNA. The RT-qPCR reactions were run in a RotorGene PCR machine using RotorGene 5.0 software. The sequence of CFTR primers was CAC ATA GCT CGG AAG GCA GCC TAT G[FAM]G at 1038 from the 3' end for the forward primer and CAC CAC AAA GAA CCC TGA GAA GAA at 1063 from 3' end for the reverse primer, producing a product of 76 bases in length. Primer sequences for NBC1 primers were CGC TGG GAT TGT GAA GAA TTG CAG [FAM]G for the forward primer starting at 1330 bases from the 3' end and TGG CGC TTT CCT CTT TAT GTC TT starting at 1360 bases from the 3' end, producing a product 78 bases in length. Primer sequence for the NKCC1 forward primer was CAC TCC CAT TCA ATT CGT CTT TCT GGA G[FAM]G and TGG ACG TGA GTT TGG AGC AC for the reverse primer. NKCC1 primers were located at 2504 and 2551 from the 3' end for the forward and reverse primers, respectively, resulting in a product 68 bases in length.

2.5 Western Blot

Western blot analysis was used to compare the expression of proteins of interest in control and PPAR γ agonist treated cells. NHBE cells used in Ussing chamber experiments were lysed, and cellular proteins were harvested and stored at -80°C. Proteins were denatured in 10% SDS solution and run in a 7.5 % polyacrylamide gel for 45 minutes. The proteins were then transferred to a nitrocellulose membrane in 20% methanol transfer buffer. The membrane was then blocked with 5% milk in a solution containing 0.1% Tween-20, 25 mM Tris base, and 125 mM NaCl (TTBS) (blocking buffer) for 1 hour at room temperature. Antibodies were diluted in blocking buffer and rocked on the membrane overnight in a coldroom. Anti-NBC1 was diluted 1:500 and both anti-CFTR and anti- β -actin were diluted 1:1000. The membrane was then washed with blocking buffer for one hour. After washing, membranes were rocked for one hour with appropriate secondary antibodies in blocking buffer. After washing with for one hour in blocking buffer and one hour in TTBS, ECL was applied and luminescence was detected using a Kodak ImageStation scanner. The intensity of detected bands was measured using the accompanying scanning software. After measurement, the membrane was stripped and re-probed with a different antibody. Mature CFTR has a molecular weight of 168 kDa, NBC1 has a molecular weight of 116 kDa, and β -actin has a molecular weight of 41.7 kDa.

2.6 Reporter Gene Assay

A reporter gene assay was used to determine if PPAR γ stimulation had an effect on CFTR promoter activity. A firefly-luciferase reporter plasmid containing either a 0.7kb or 3.9kb CFTR promoter was used to quantify CFTR promoter activity in the presence or absence of PPAR γ agonists. A transfection control plasmid that contains a *Renilla* luciferase conjugated to a Herpes-simplex virus thymidine kinase promoter, pRL-TK (Promega, Ont), was co-transfected with the CFTR plasmids to control for transfection efficiency. A549 cells grown on 24 well plates were cultured until 60% confluency and then transfected using Effectene transfection reagent. Transfection followed the standard protocol from Qiagen using 0.2 μ g of CFTR plasmid, 0.01 μ g pRL-TK plasmid, 1.6 μ l Enhancer, and 5 μ l Effectene reagent per well in growth media. The media containing transfection reagent was left on cells overnight to allow maximal absorption of plasmid DNA. Transfection media was then removed and replaced with normal growth media or growth media containing PPAR γ agonists. After 24 hours of exposure to PPAR γ agonists, cells were washed with phosphate-buffered saline 3 times and then lysed using Passive Lysis Buffer from the Dual-Luciferase Assay kit (Promega). 20 μ l of cell lysate was added to 100 μ l of firefly luciferase reaction mix and luminescence was detected using a Turner Designs (Sunnyvale, CA) TD-20/20 luminometer. The firefly reaction was quenched and the *Renilla* reaction was initiated using 100 μ l of Stop and Glo[®] reagent and measured in the luminometer. Luminescence was recorded for 3 10-second intervals for each luciferase reaction. The values for firefly reaction were nearly constant and were averaged over the 30-second interval. Since the

Renilla reaction deteriorated at a much quicker rate than the firefly luciferase reaction, only the first 10-second interval value was used for correction.

2.7 Statistical Analysis

All values are expressed as mean $\pm$ standard error unless otherwise stated. Both peak and steady-state responses were used to compare the responses of NHBE cells to ion transport modulators. ANOVA was used to compare the means of multiple groups, with significance considered to be $p\text{-value} < 0.05$ in the Tukey post-test. Student's t-Test was used to compare the difference between two groups with statistical significance considered to be a $p\text{-value} < 0.05$. Significant digits were determined using purely statistical calculations based on the recorded results.

Chapter 3 Results

3.1 Ussing Chamber Results

3.1.1 Intact Cell Experiments

3.1.1.1 Experiments in Normal KHS

Experiments with NHBE cells were initially run in normal KHS. The baseline characteristics of control and pioglitazone-treated cells are shown in Table 3.1. There is a significant difference between the basal SCC between control cells and those treated with pioglitazone ($p<0.01$). The same percentage of initial SCC was inhibited by amiloride in pioglitazone-treated cells as in controls ($p=0.96$). Transepithelial resistance was not affected by pioglitazone treatment ($p=0.18$). Cells were then treated with a variety of agonist molecules to activate anion secretion. Bilateral forskolin, basolateral carbachol, NECA, and ATP, with NECA and ATP used independently on both the apical and basolateral membranes, were all used to activate anion secretion in NHBE cells. Inhibitor studies used cells pre-treated with forskolin, as the steady-state response is higher and more stable than the other activators. Inhibitors used include BaCl_2 , furosemide, clotrimazole, XE991, and tetrapentylammonium chloride. $\text{PPAR}\gamma$ agonists used include pioglitazone and GW1929.

Treatment	Basal Current ($\mu\text{A}/\text{cm}^2$)	Transepithelial Resistance (Ω)	Amiloride Response (% Basal SCC)	n
Control	22.4 ± 1.8	370 ± 22	71.5 ± 4.4	n=24
Pioglitazone	$16.0\pm 1.1^{**}$	450 ± 56	69.9 ± 3.1	n=24

Table 3.1. Summary of baseline characteristics and amiloride responses of NHBE cells after treatment with pioglitazone. $^{**}p<0.01$

In NHBE cells activated with bilateral forskolin, anion secretory responses were significantly lowered in $\text{PPAR}\gamma$ agonist treated cells. Representative SCC responses to

forskolin are shown in Figure 3.1.2A. Control cells had a forskolin response of $37.8 \pm 2.2 \mu\text{A}/\text{cm}^2$ ($n=13$), pioglitazone treated cells had responses of $26.0 \pm 1.4 \mu\text{A}/\text{cm}^2$ ($n=11$) ($p < 0.001$), and the GW1929-treated forskolin response was $27.7 \pm 1.6 \mu\text{A}/\text{cm}^2$ ($n=3$) ($p=0.052$) as shown in Figure 3.1.1B. Forskolin directly activates adenylate cyclase, increasing cAMP levels and activating ion transport by direct interaction or phosphorylation of CFTR and other ion transport proteins. This decrease in anion secretion could be produced by many factors and further experiments were used to examine both apical and basolateral membrane factors.

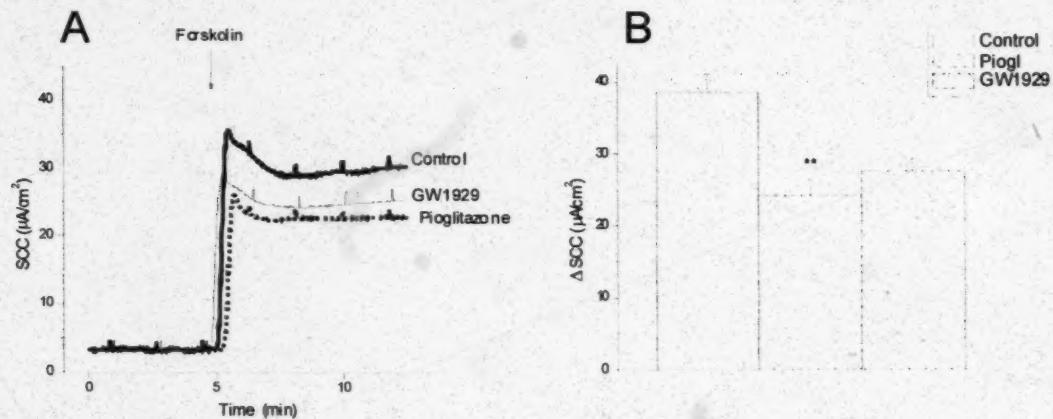


Figure 3.1.1. (A) Representative recordings showing forskolin response in control, pioglitazone-treated, and GW1929-treated monolayers. Forskolin increases cytosolic cAMP and increases anion secretion through CFTR. (B) SCC response to forskolin in NHBE monolayers treated with pioglitazone and GW1929. ** $p < 0.01$.

Membranes were washed with KHS and another set of experiments was performed to examine the SCC response to carbachol. The carbachol response observed was biphasic in nature for all treatments. As seen in figure 3.1.2A, an initial spike occurred immediately after addition of carbachol to the basolateral chamber, followed by a delayed increase in anion secretion. The initial response values were $9.6 \pm 1.0 \mu\text{A}/\text{cm}^2$

(n=5) for controls, $7.7 \pm 0.9 \mu\text{A}/\text{cm}^2$ (n=4) for pioglitazone-treated, and $6.2 \pm 0.6 \mu\text{A}/\text{cm}^2$ (n=3) for GW1929 treated cells and did not reach statistical significance ($p > 0.05$). The second-phase peak response values were significantly different between controls and PPAR γ agonist treated cells. The second phase response of control cells was $15.7 \pm 2.5 \mu\text{A}/\text{cm}^2$, pioglitazone-treated cells had response of $0.9 \pm 0.9 \mu\text{A}/\text{cm}^2$ ($p < 0.01$) and GW1929 treated cells had a response of $2.0 \pm 0.3 \mu\text{A}/\text{cm}^2$ ($p < 0.01$). The steady-state responses did not reach statistical difference – 8.8 ± 1.3 , 5.1 ± 0.5 , and $4.5 \pm 0.4 \mu\text{A}/\text{cm}^2$ respectively ($p > 0.05$). This result suggests that there is a change in the signaling pathway that produces the reduction in the second-phase carbachol response. Since carbachol and M $_3$ receptors act through elevation of intracellular calcium, one possible explanation is reduction in calcium release from intracellular stores.

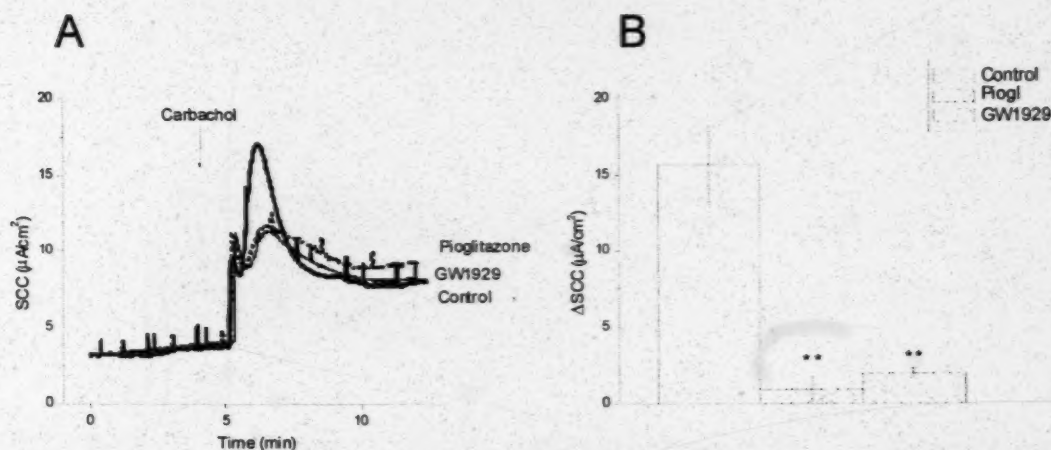


Figure 3.1.2. (A) Representative SCC responses to carbachol in NHBE monolayers. Carbachol activates muscarinic receptors on the basolateral membrane to activate Ca^{2+} -dependent anion secretion. (B) Control cells displayed a significantly larger secondary peak in SCC response to carbachol than either GW1929 or pioglitazone-treated cells. ** $p < 0.01$.

Adenosine and ATP receptors are expressed on the apical and basolateral membranes of NHBE cells. To investigate any effect PPAR γ stimulation these receptors,

NECA and ATP were used to stimulate anion secretion. With receptor expression occurring at both membranes, cells were treated with agonist on the apical membrane, followed with basolateral stimulation after 10 minutes. The apical response to NECA was $8.9 \pm 1.6 \mu\text{A}/\text{cm}^2$ ($n=4$) in controls and $9.0 \pm 1.0 \mu\text{A}/\text{cm}^2$ ($n=3$) in pioglitazone-treated monolayers. The basolateral response to NECA was $34.7 \pm 0.9 \mu\text{A}/\text{cm}^2$ and $35.4 \pm 3.8 \mu\text{A}/\text{cm}^2$ respectively. NECA response was not statistically different for both the apical and basolateral membranes. As P1 receptors activate cAMP production, this result contradicts the observations using forskolin. Apical NECA responses are shown in Figure 3.1.3 and basolateral responses are shown in Figure 3.1.4.

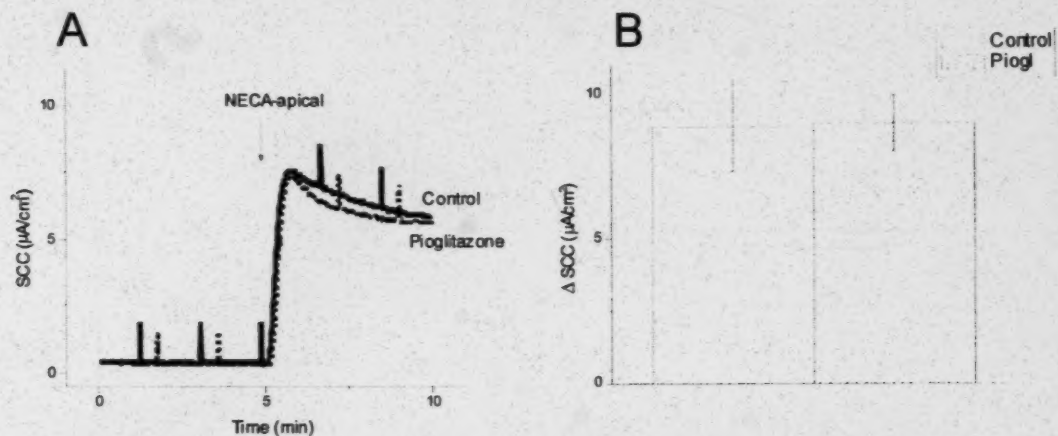


Figure 3.1.3. (A) Representative SCC responses to NECA applied to the apical membrane. NECA activates adenosine receptors in the apical membrane and stimulates cAMP-dependent anion secretion. (B) Summary of apical NECA responses.

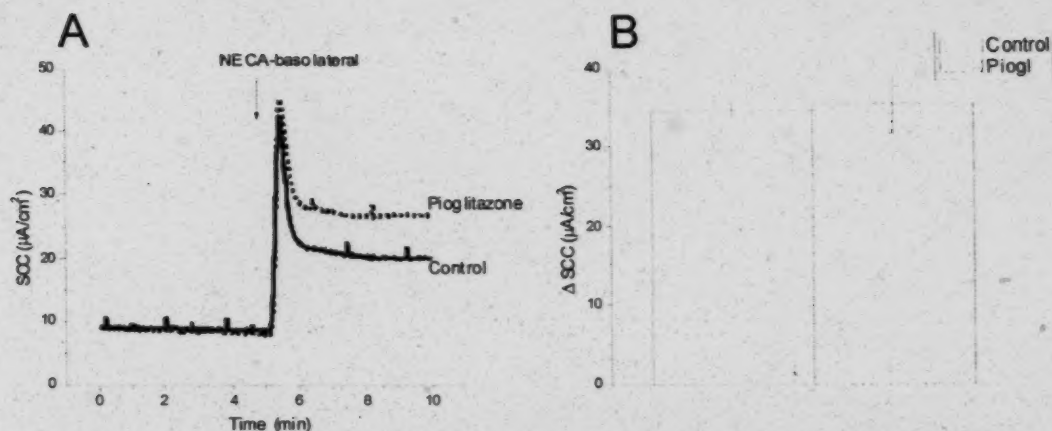


Figure 3.1.4. (A) Representative recordings of basolateral responses to NECA after apical NECA. NECA activates cAMP-dependent anion secretion. (B) Summary of results of SCC response to basolateral NECA.

The apical ATP response was higher in pioglitazone-treated cells than in controls ($p < 0.01$) at $33.2 \pm 1.6 \mu\text{A}/\text{cm}^2$ ($n=4$) and $20.2 \pm 2.4 \mu\text{A}/\text{cm}^2$ ($n=4$), respectively. SCC responses to apical ATP are shown in Figure 3.1.5. Upon further examination of the apical response, a second peak is observed. This peak is diminished in PPAR γ treated cells as seen in Figure 3.1.6. This second phase resulted in significantly different increases in SCC of $2.1 \pm 0.7 \mu\text{A}/\text{cm}^2$ pioglitazone-treated and $6.9 \pm 0.3 \mu\text{A}/\text{cm}^2$ in control cells ($p < 0.001$). Basolateral ATP response was also affected by PPAR γ stimulation – in an opposite manner to the apical response. Pioglitazone treatment resulted in a basolateral ATP response of $3.2 \pm 0.7 \mu\text{A}/\text{cm}^2$, while control membrane response was $26.7 \pm 4.1 \mu\text{A}/\text{cm}^2$ ($p < 0.01$). Basolateral ATP responses are summarized in Figure 3.1.7. This paradoxical response between membranes may be the result of the differential regulation of signaling pathways. The basolateral ATP receptor activates the calcium-dependent signaling pathway – similar to carbachol. This alteration in basolateral ATP response is most likely related to the changes observed in the carbachol response, due to the common downstream signaling pathway. The apical ATP receptor only activates the

calcium-dependant signaling pathway. However, the alteration of the both phases of the biphasic apical ATP response suggests an interesting difference following PPAR γ treatment.

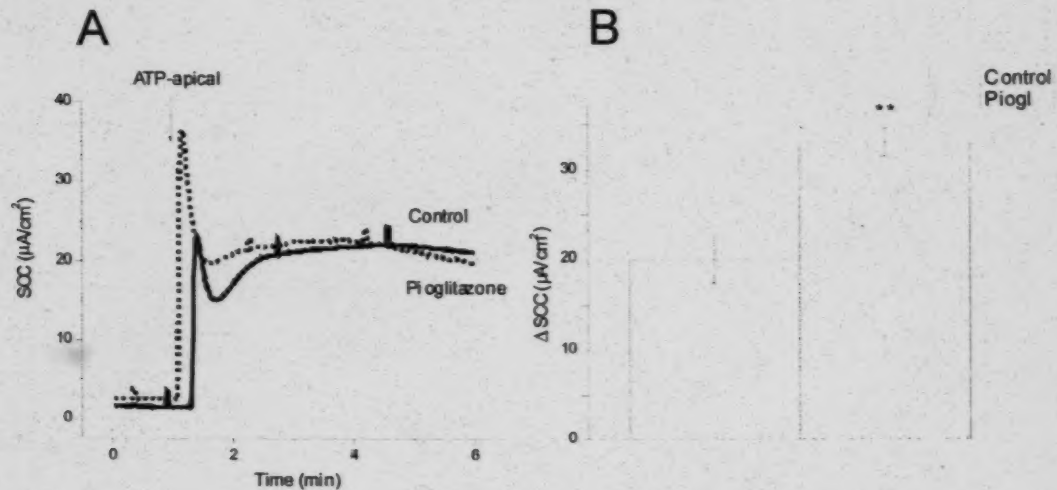


Figure 3.1.5. (A) Typical response to ATP applied to the apical solution. ATP activates P2 receptors in the apical membrane to increase anion secretion through both cAMP and Ca^{2+} -dependent mechanisms. (B) Summary of initial phase of the apical ATP response. ** $p < 0.01$.

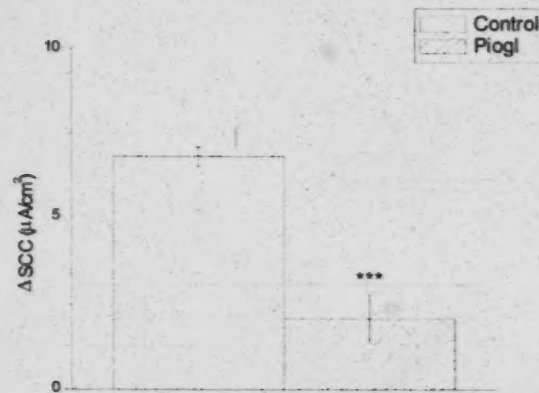


Figure 3.1.6. Summary of the second-phase apical ATP response. *** $p < 0.001$.

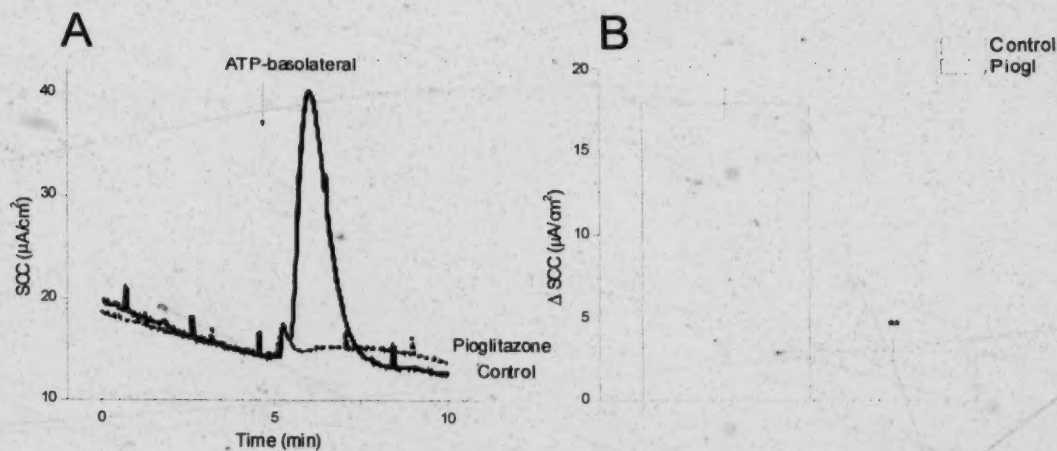


Figure 3.1.7. (A) Typical response of NHBE monolayers to ATP in the basolateral solution. ATP activates P2 purinergic receptors in the basolateral membrane to activate Ca^{2+} -dependent anion secretion. (B) Summary of peak SCC response to basolateral ATP administration. $**p < 0.01$.

To examine the difference in anion secretion activated by the calcium-dependent signaling pathway after PPAR γ agonist treatment, a series of calcium-pathway experiments were performed. Thapsigargin, an inhibitor of the sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA), was used to evaluate pioglitazone-induced changes in intracellular calcium handling. Thapsigargin activated currents were significantly different in control and pioglitazone-treated cells at $9.6 \pm 1.3 \mu\text{A}/\text{cm}^2$ ($n=5$) and $5.5 \pm 1.0 \mu\text{A}/\text{cm}^2$ ($n=6$) respectively ($p < 0.05$). Two hypotheses explain this phenomenon in pioglitazone-treated cells: less calcium is released from intracellular stores or less calcium is entering via the plasma membrane. The peak SCC responses of anion secretion activators are summarized in Table 3.2.

Activator	Control ($\mu\text{A}/\text{cm}^2$)	Pioglitazone ($\mu\text{A}/\text{cm}^2$)	GW1929 ($\mu\text{A}/\text{cm}^2$)
Forskolin	37.8 $\pm$ 2.2 (n=13)	26.0 $\pm$ 1.4 (n=11) **	27.7 $\pm$ 1.6 (n=3)
Peak-Carbachol	19 $\pm$ 3.0 (n=8)	7.9 $\pm$ 0.9 (n=4) *	6.4 $\pm$ 0.4 (n=3) *
ATP-apical	20.2 $\pm$ 2.4 (n=4)	33.2 $\pm$ 1.6 (n=4) **	N/A
ATP-basolateral	26.7 $\pm$ 4.1 (n=4)	3.2 $\pm$ 0.7 (n=4) **	N/A
NECA-apical	8.9 $\pm$ 1.6 (n=4)	9.0 $\pm$ 1.0 (n=3)	N/A
NECA-basolateral	34.7 $\pm$ 0.9 (n=4)	35.4 $\pm$ 3.8 (n=3)	N/A
Thapsigargin	9.6 $\pm$ 1.3 (n=5)	5.5 $\pm$ 1.0 (n=6) *	N/A

Table 3.2. Summary of SCC responses to activators of secretion. * $p < 0.05$; ** $p < 0.01$.

Inhibitor responses were used to investigate possible basolateral transport contributions to the decreases in both forskolin and carbachol responses. Preliminary data using furosemide showed no difference between control and PPAR γ agonist treated and was dropped from further SCC studies. Barium chloride added to the basolateral chamber did not reveal any statistical difference in overall potassium channel contribution to anion secretion. However, the lack of selectivity of this inhibitor requires the exploration of individual potassium currents, since several different potassium conductances have been identified in the airways. XE991, clotrimazole, tetrapentylammonium chloride, and tolbutamide were used to isolate the contributions of these potassium currents. After preliminary experiments, tolbutamide was removed from the protocol, as it had no effect on anion secretion in NHBE cells. Clotrimazole had a minor effect when compared to XE991 or TPAC. Table 3.3 shows the inhibitor responses of NHBE cells after forskolin stimulation. There was no significant difference in any of the inhibitor responses after pioglitazone treatment when compared to control. It is possible that the decrease in forskolin current in NHBE cells after PPAR γ stimulation is due to a greater proportion of basolateral potassium channels in an open-state in PPAR γ agonist treated cells than in untreated cells under basal conditions. To

test this hypothesis, a basolateral membrane only preparation was used as described in

3.1.3.

Inhibitor	Control ($\mu\text{A}/\text{cm}^2$)	Pioglitazone ($\mu\text{A}/\text{cm}^2$)
BaCl ₂	-28.3 $\pm$ 2.9 (n=5)	-22.2 $\pm$ 1.0 (n=5)
TPAC	-19.9 $\pm$ 1.1 (n=3)	-19.8 $\pm$ 1.5 (n=3)
Clotrimazole	-4.3 $\pm$ 1.8 (n=2)	-2.1 $\pm$ 1.0 (n=4)
Xe991	-8.6 $\pm$ 0.1 (n=2)	-8.5 $\pm$ 0.7 (n=4)

Table 3.3. Effect of ion transport inhibitors on SCC after bilateral forskolin treatment.

3.1.1.2 Experiments in Low-Chloride KHS

To determine the chloride ion contribution to the anion flux observed in normal KHS solution experiments, a low-chloride KHS buffer was employed. Basal currents for control and pioglitazone-treated cells were not significantly different at 4.9 ± 0.8 and $5.6\pm1.0 \mu\text{A}/\text{cm}^2$ respectively. The forskolin responses in lcKHS were minute compared to KHS – suggesting chloride is the major anion secreted by NHBE cells. However, similar to experiments performed in normal KHS, pioglitazone-treated cells had a significantly lower increase in SCC compared to controls at $5.1\pm0.3 \mu\text{A}/\text{cm}^2$ (n=4) and $7.1\pm0.2 \mu\text{A}/\text{cm}^2$ (n=3) respectively ($p<0.01$). Data from lcKHS forskolin responses are shown in figure 3.1.8. Since the difference in anion secretion between control and PPAR γ agonist treated cells remains in lcKHS, this suggests that the alteration in secretion of anions is a result of increased intracellular cAMP is chloride-independent and may be caused by changes in secretion of bicarbonate ions.

The carbachol responses in lcKHS were not significantly different between control and pioglitazone treated at 2.8 ± 0.4 (n=4) and $2.4\pm0.3 \mu\text{A}/\text{cm}^2$ (n=3) respectively (Figure 3.1.9B). Representative responses to carbachol in lcKHS are shown in Figure

3.1.9A. This finding has three major implications. First, that the difference in carbachol response after PPAR γ agonist treatment is the result of a decrease in chloride ion secretion. Second, that the cAMP and calcium-dependent secretory pathways are regulated differently by PPAR γ activation. Finally, NHBE cells are capable of selectively secreting anions.

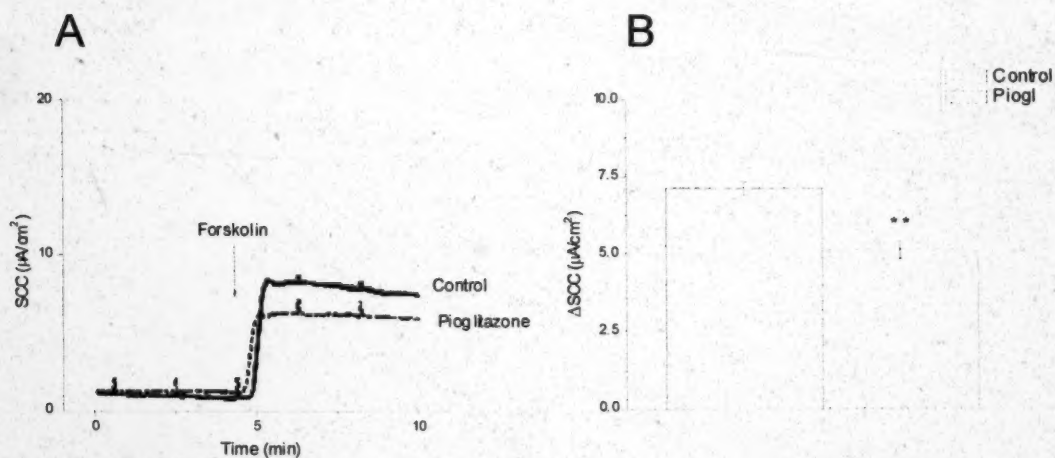


Figure 3.1.8. (A) Representative NHBE responses to forskolin in low-chloride KHS. (B) Summary of forskolin responses in lKHS. ** $p < 0.01$.

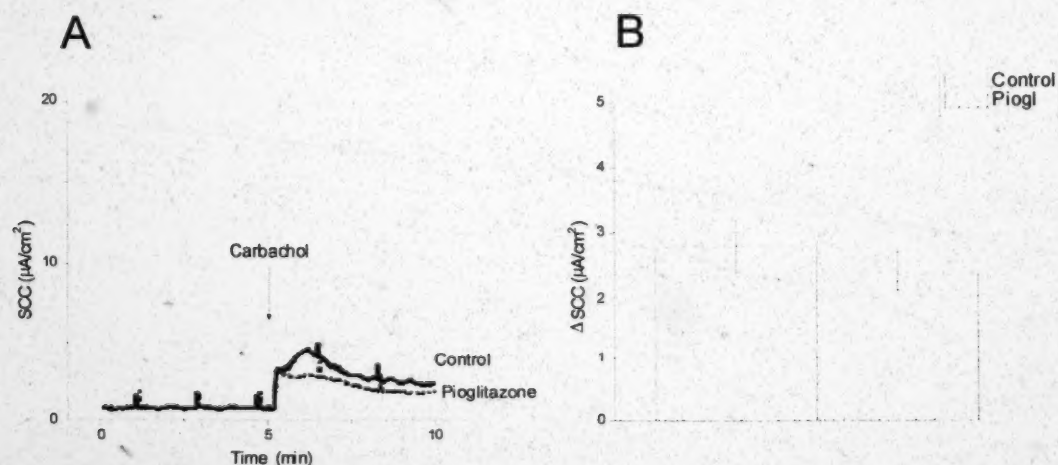


Figure 3.1.9. (A) Representative NHBE monolayer responses to carbachol in low-chloride KHS. (B) Summary of carbachol responses in lKHS.

3.1.1.3 Experiments in Bicarbonate-Free KHS

Bicarbonate-free KHS was used to identify the HCO_3^- contribution to anion secretion in response to forskolin and carbachol stimulation. The basal current was significantly higher in pioglitazone treated cells in bfKHS ($10.6 \pm 1.5 \mu\text{A}/\text{cm}^2$ vs. $6.3 \pm 0.3 \mu\text{A}/\text{cm}^2$) ($p < 0.05$). This significance disappears after treatment with apical amiloride ($p = 0.73$), suggesting an alteration in sodium handling in PPAR γ -treated cells in bicarbonate-free buffer. Upon forskolin-stimulation, both control and pioglitazone treatment displayed similar responses of $28.0 \pm 4.5 \mu\text{A}/\text{cm}^2$ ($n = 3$) and $28.6 \pm 2.5 \mu\text{A}/\text{cm}^2$ ($n = 3$) respectively. The NHBE responses to forskolin in bfKHS are summarized in Figure 3.1.10. The peak pioglitazone response in normal KHS was $24.2 \pm 2.2 \mu\text{A}/\text{cm}^2$, this value was not significantly different from the bfKHS response of control or pioglitazone-treated NHBE monolayers (ANOVA). Pioglitazone treatment impairs the selective secretion of bicarbonate ions upon stimulation of intracellular cAMP levels with forskolin. The difference in peak carbachol response in NHBE cells was unaffected by the bfKHS, with control SCC responses of $30.0 \pm 5.90 \mu\text{A}/\text{cm}^2$ and pioglitazone-treated responses of $6.6 \pm 2.0 \mu\text{A}/\text{cm}^2$ ($p < 0.05$). Carbachol response in bfKHS is summarized in Figure 3.1.10.

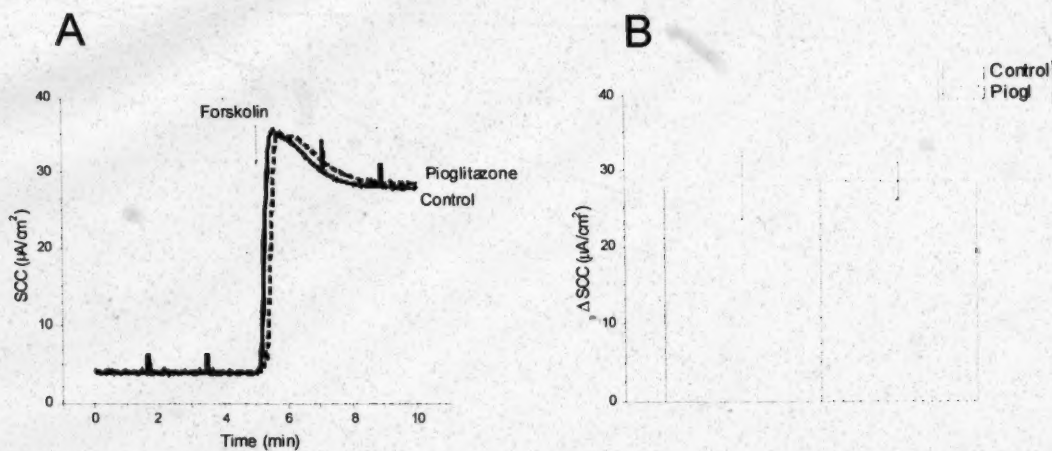


Figure 3.1.10. (A) Representative responses to forskolin in bicarbonate-free KHS. (B) Summary of NHBE responses to forskolin in bfKHS.

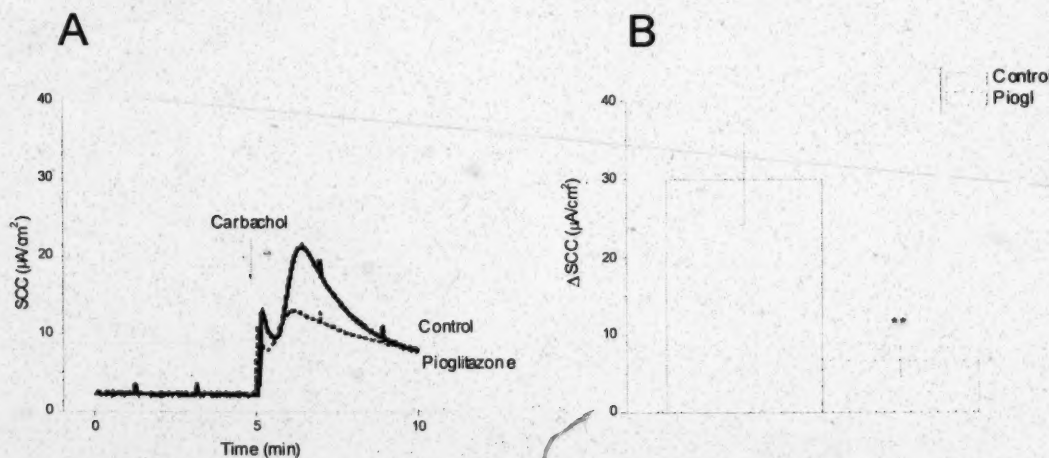


Figure 3.1.11. (A) Representative carbachol responses from NHBE monolayers in bicarbonate-free KHS. (B) Summary of peak SCC responses to carbachol in bfKHS. ** $p < 0.01$.

3.1.2 Apical Membrane Studies

To examine the possible alterations in apical membrane anion conductance, a modified Ussing chamber experiment was employed. High potassium KHS (bdKHS) was used to depolarize the basolateral membrane, producing an apical to basolateral

chloride gradient. Amiloride and barium chloride were added to the apical solution to ensure that only anion movement is responsible for the observed SCC. After forskolin stimulation to activate anion absorption, control cells displayed an SCC of $-43.0 \pm 5.02 \mu\text{A}/\text{cm}^2$, pioglitazone treated cells had an SCC of $-54.0 \pm 2.82 \mu\text{A}/\text{cm}^2$, and GW1929 responses were $-50.9 \pm 3.59 \mu\text{A}/\text{cm}^2$. There was no statistical difference in forskolin response due to pioglitazone ($p=0.12$) or GW1929 ($p=0.31$), suggesting that changes apical anion conductance are not involved in alterations in observed differences to overall anion conductance. However, a general trend was observed that PPAR γ agonists increased the forskolin response, suggesting that apical anion conductance is indeed increased by stimulation of PPAR γ . Representative traces from apical membrane studies are shown in Figure 3.1.11.

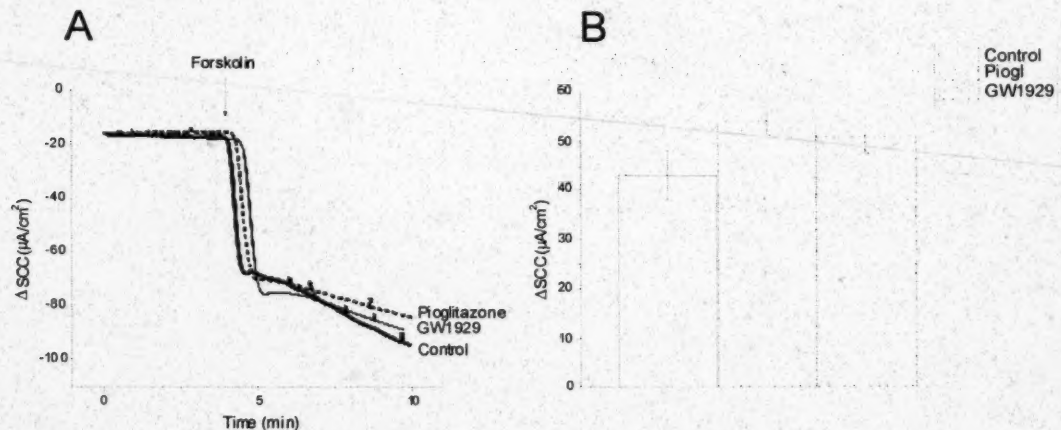


Figure 3.1.11. Apical membrane response to forskolin. The basolateral membrane of NHBE monolayers was depolarized with bdKHS and forskolin was applied.

3.1.3 Basolateral Membrane Studies

3.1.3.1 Bicarbonate Studies

To investigate the effect of PPAR γ stimulation on the expression of NBC1, another modified Ussing chamber experiment was employed. The apical membrane was permeabilized with nystatin, ouabain was added to the basolateral chamber, and solutions with altered sodium concentrations were employed. A 10 mM sodium gluconate solution was used apically and a 50 mM sodium gluconate solution was used in the basolateral chamber. A voltage clamp was applied from +50 to -50 mV and a steady-state SCC was measured every 10 mV with the clamp performed before and after 2 mM DNDS addition to the basolateral membrane. The averaged results of the voltage clamp experiments are shown in Figure 3.1.12. DNDS treatment produced a difference in the SCC observed in the controls cells during voltage clamp, but did not produce a difference in pioglitazone-treated cells. PPAR γ agonists reduce the current attributed of the basolateral membrane sodium bicarbonate co-transporter NBC1 compared to control in NHBE cells.

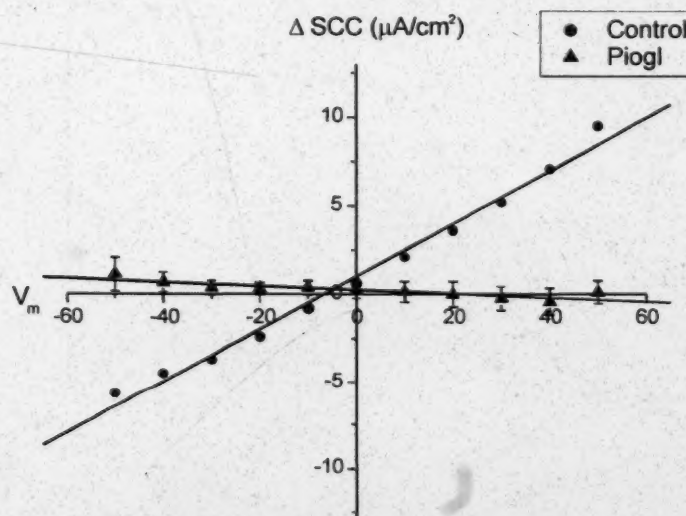


Figure 3.1.12. I-V plot of the DNDS-sensitive current in NHBE monolayers in the presence of a basolateral to apical sodium gradient. Control (n=1) had a larger DNDS-sensitive current than pioglitazone-treated (n=4) monolayers.

3.1.3.2 Potassium Channel Studies

To investigate the hypothesis that PPAR γ stimulation causes an increase in the number of potassium channels in the open-state under basal conditions, an isolated basolateral membrane experiment was performed. Ouabain was added to the basolateral chamber to inhibit the electrogenic Na⁺/K⁺ ATPase on the basolateral membrane. The apical membrane of the NHBE cells was permeabilized with nystatin in the presence of an apical to basolateral potassium gradient. This resulted in an increase in SCC in both pioglitazone-treated and control cells, corresponding to absorption of K⁺ ions across the basolateral membrane. After steady-state was reached, BaCl₂ was added to non-selectively inhibit basolateral potassium channels. The SCC inhibited by barium corresponds to the potassium current under basal conditions. Control cells showed a barium response of $-0.87 \pm 0.27 \mu\text{A}/\text{cm}^2$ ($n=3$), while pioglitazone-treated cells had an SCC response of $-2.6 \pm 0.88 \mu\text{A}/\text{cm}^2$ ($n=5$). There was no statistical difference in baseline potassium current in control and PPAR γ agonist treated cells ($p=0.13$). PPAR γ activation does not affect the proportion of basolateral potassium channels in the open-state under basal conditions.

3.2 Quantitative PCR Studies

To examine the expression of proteins of interest in NHBE cells at the mRNA level, quantitative reverse-transcriptase PCR was employed. FAM-conjugated primers produce a signal upon extension that is detected and measured. As the number of copies of a particular mRNA increase, the fluorescent signal also increases, allowing a quantitative measure of mRNA. To calibrate the observed fluorescence signal, another

primer for a "housekeeping gene" conjugated to JOE dye is used and the ratio between the two allows comparison of mRNA transcription. The expression levels of NBC1 and CFTR were examined using intron-spanning primers. RT-qPCR experiments were performed using 20, 30, and 40 ng of cytosolic RNA. The results of the PCR experiments are displayed in Figure 3.21. RNA isolated from control cells had a normalized cycle threshold (Ct) of 29.3 ± 0.15 (n=9), pioglitazone-treated cells had a count of 27.1 ± 0.30 (n=9), GW1929-treated cells had a Ct of 28.2 ± 0.05 (n=9), and the "no template control" (NTC) had a count of 38 ± 0.20 (n=3) for CFTR. A lower Ct indicates higher expression of mRNA, suggesting that CFTR mRNA was upregulated by PPAR γ agonists. NBC1 mRNA expression is shown in Figure 3.2.2. Control cells had a normalized Ct of 21.9 ± 0.16 (n=6), while pioglitazone-treated cells had a normalized Ct of 22.9 ± 0.14 (n=6). NTC had a count of 34.8 (n=1). NKCC1 expression was also examined using PCR. The expression of NKCC1 mRNA was unchanged by treatment with pioglitazone (p=0.86), with Cts of 22.9 ± 0.66 for control (n=3), 22.8 ± 0.16 (n=3) for pioglitazone-treated cells, and 31.4 for NTC (n=1). Results of NKCC1 quantitative PCR are shown in Figure 3.2.3.

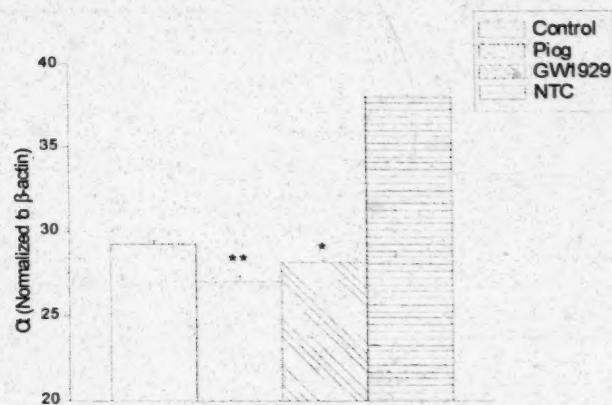


Figure 3.2.1. CFTR mRNA expression after PPAR γ agonist treatment. Values are expressed as cycle number normalized to β -actin. Decreased count number indicates an increase in CFTR mRNA expression. * $p < 0.05$; ** $p < 0.01$.

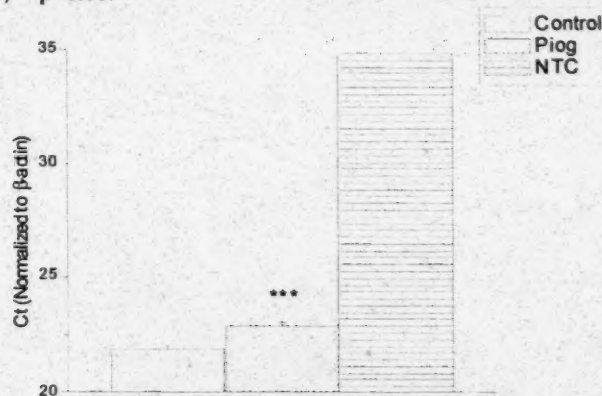


Figure 3.2.1. NBC1 mRNA expression after PPAR γ agonist treatment. Values are expressed as cycle number normalized to β -actin. Increased count number indicates an decrease in NBC1 mRNA expression. *** $p < 0.001$.

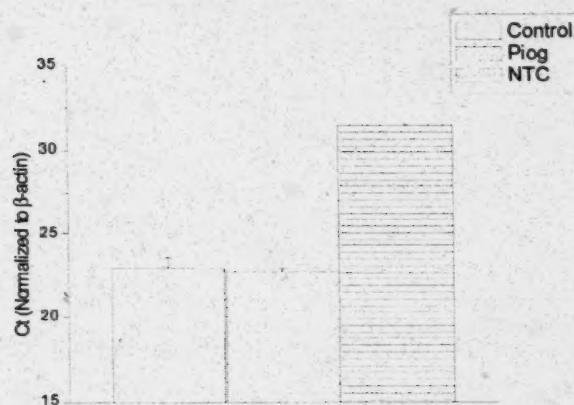


Figure 3.2.3. Quantification of NKCC1 mRNA in NHBE cells treated with PPAR γ agonists. Values are expressed as count normalized to β -actin. No difference was observed in NKCC1 expression between control and treated cells.

3.3 Western Blot studies

To verify the functional and RT-qPCR results, western blot technique was used to investigate the cellular expression of proteins of interest. CFTR is the major protein responsible for anion conductance in the apical membrane of NHBE cells. CFTR mRNA was also upregulated in PPAR γ agonist treated cells. The total cellular expression of CFTR was examined in control and pioglitazone treated NHBE cells. CFTR detected with a monoclonal mouse antibody is displayed in figure 3.3.1. Quantification of the CFTR western blot resulted in 0.243 Arbitrary Units (AU) for control and 0.246 AU for pioglitazone-treated. Due to the faintness of observed staining, immature CFTR was not visible on the blot.

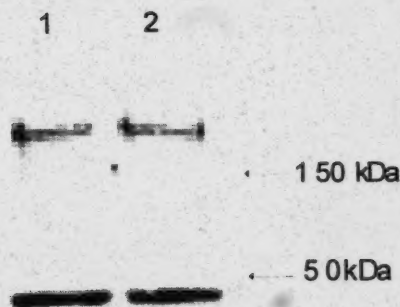


Figure 3.3.1. Expression of CFTR protein in NHBE cells treated with pioglitazone. Lane 1 shows CFTR staining in control cells and Lane 2 shows CFTR in pioglitazone treated cells. The band near 150 kDa shows CFTR staining and the band near 50 kDa shows actin staining.

NBC1 functional data suggested downregulation and verification using western blot was necessary. The expression of NBC1 was made using a polyclonal rabbit antibody and shown in Figure 3.3.2. NBC1 quantification revealed 0.394 AU for control and 0.187 AU for pioglitazone-treated. β -actin was detected using a polyclonal rabbit antibody and used to calibrate the quantification of both NBC1 and CFTR. The detection of similar CFTR protein levels in control and pioglitazone treated cells is contrary to the results obtained using RT-qPCR – a result that at present is not clearly explained. It is possible that a homeostatic mechanism prevents increase expression of CFTR in NHBE cells.

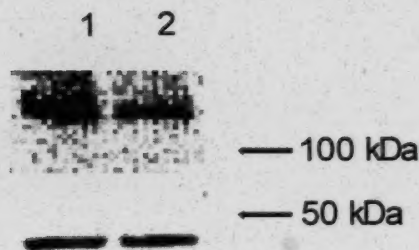


Figure 3.2.2. Expression of NBC1 in NHBE cells. Lane 1 contains protein from control cells; Lane 2 shows NBC1 expression in pioglitazone-treated cells. The band visible above 100 kDa corresponds to NBC1 staining, while the band near 50 kDa corresponds to β -actin.

3.4 Promoter Studies

To explain the difference between the western blot and RT-qPCR studies of CFTR expression after PPAR γ stimulation, a promoter study was used. Two firefly luciferase conjugated CFTR promoter plasmids were employed – a truncated 0.7 kb promoter and a larger 3.9 kb promoter. The luminescence from firefly luciferase was calibrated with luminescence produced by a co-transfected *Renilla* luciferase plasmid. The ratio of the firefly to *Renilla* was used to compare changes in CFTR promoter activity with data expressed as Fold Increase versus control, a ratio to the luminescence of control cell lysate. A549 cells were used in these experiments because they are more easily cultured and transfected than NHBE cells, while still containing the cellular machinery required for the assay. Controls were assigned a value of 1.00 ± 0.13 ($n=6$) for the 3.9kb promoter and 1.00 ± 0.17 ($n=7$) for the 0.7kb promoter. Pioglitazone treatment increased the expression of firefly luciferase in the 3.9 kb promoter, but had reduced effectiveness in the 0.7 kb truncated CFTR promoter – 1.68 ± 0.06 ($n=5$) fold increase ($p < 0.001$) and 1.28 ± 0.25 ($n=5$) fold increase ($p < 0.05$) respectively. A novel PPAR γ

agonist, FMOC-L-leucine, was also used to treat the transfected cells. FMOC-L-leucine increased expression of both promoters, but the 0.7 kb promoter activity of 1.89 ± 0.25 (n=6) fold increase ($p < 0.001$) was reduced activity compared to the 3.9 kb promoter at 3.03 ± 0.27 (n=7) fold increase versus control ($p < 0.001$). The activity of the CFTR reporter plasmids in response to PPAR γ agonists is summarized in Figure 3.4. These studies show that PPAR γ stimulation increases the transcriptional activity of the CFTR promoter and downstream protein levels in A549 cells. This parallels the results seen in the RT-qPCR studies, but contradicts the results of functional and western blot studies in NHBE cells. These results have been confirmed by Dr. Mitchell Drumm's laboratory (Case Western Reserve University, OH). The results produced by Dr. Drumm's laboratory will be included in the manuscript for publication.

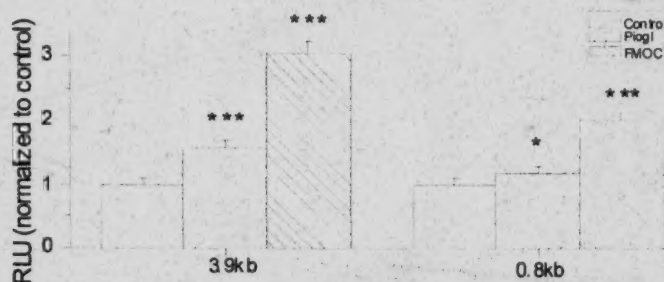


Figure 3.4. Relative activity of CFTR promoter plasmids. Data is expressed as RLU normalized to control. * $p < 0.05$; *** $p < 0.001$.

Chapter 4 Discussion

4.1 Physiological Roles of PPAR γ

Peroxisome Proliferator Activated Receptor subtype- γ is a member of the nuclear receptor family that activates or suppresses transcription of target genes upon activation. Upon ligand binding, PPAR γ heterodimerizes with the RXR receptor and binds to PPAR response elements (PPRE) in the cell nucleus (51). The consensus PPRE half-site is AGGTCA, although other variations have been identified in humans and other mammalian species (39). There are many pharmacological activators of PPAR γ , but unlike other nuclear receptors, there is no clear agonist molecule. Several different molecules have been proposed as agonists, but the consensus is that PPARs are endogenously activated by alterations in free-fatty acid (FFA) concentrations and changes in cellular energy (19, 59). Expression of the different PPAR subtypes is widespread throughout the body, but PPAR γ is expressed mainly in the liver and adipose tissue. PPAR γ is known to regulate differentiation of adipocytes and lipid metabolism. Interestingly, this receptor is also found in the lung epithelium (83). As epithelial transport is a highly energetic process, PPARs may have important regulatory roles at the transcriptional level. In addition to possible roles in regulation of ion transport, some PPAR γ agonists have been shown to possess anti-inflammatory properties (18, 67). These properties of PPAR γ agonists provide a potential dual-mechanism for airway disease therapy. This thesis examines the effects of PPAR γ stimulation on ion transport and evaluates the potential benefits and risks of use of PPAR γ agonists therapeutically.

Specifically, this thesis addresses possible applications of PPAR γ agonists in lung disease.

4.2 PPAR γ as a Regulator of Ion Transport

The focus of this thesis was on PPAR γ as a potential regulator of ion transport in normal human bronchial epithelia. The activation and inhibition of ion secretion and absorption with pharmacological agents allowed a comparison of ion transport properties of control cells and cells treated with PPAR γ agonists. In general, the PPAR γ agonist treated monolayers displayed a reduced response to secretagogues. As PPARs are regulators of gene transcription, several different proteins were affected by treatment.

Basal ion transport characteristics of NHBE monolayers were affected by PPAR γ agonist treatment. Basal current was reduced in pioglitazone-treated cells, while the percentage of SCC inhibited by amiloride was not affected. This result suggests that sodium channels are not affected by PPAR γ agonist treatment. The decrease in basal current may be a result of several factors and may be explained by experiments discussed below.

PPAR γ agonists reduced the increase in SCC due to forskolin treatment.

Forskolin activates cAMP production by activating adenylate cyclase and, as a result, increases anion secretion through apical chloride channels – CFTR in particular. This reduction was also observed when chloride was removed from the bathing solution, suggesting that bicarbonate ions were responsible for the difference between control and PPAR γ agonist-treated cells. This result was confirmed by removal of bicarbonate from the bathing solution. There was no difference in SCC between control and pioglitazone-

treated cells in bfKHS and pioglitazone treated cells in normal KHS. Observations in Calu-3 cells using radioactive flux measurements have shown that forskolin activated SCC is entirely due to bicarbonate ion secretion. SCC was still observed after bicarbonate removal from solution, suggesting that HCO_3^- secretion is not responsible for the majority of anion secretion activated forskolin in NHBE monolayers.

As bicarbonate secretion is affected, exploration of PPAR γ effects on NBC1 was necessary. Voltage-clamp experiments showed a reduction in DNDS-sensitive current after PPAR γ treatment. Western blot analysis confirmed a decrease in NBC1 expression in NHBE monolayers. The reduction in NBC1 does not explain the reduction in bicarbonate-dependent anion secretion due to forskolin. NBC1 is regulated by cAMP and addition of forskolin may decrease the influx of bicarbonate through NBC1 in the kidney (64). However, other proteins involved in forskolin-dependent secretion may be affected. It is possible that carbonic anhydrase expression is reduced and less carbon dioxide is converted to bicarbonate within the NHBE cells – this possibility was not examined in this thesis. This hypothesis becomes more likely when considering the effect of forskolin in bfKHS. The total removal of bicarbonate and carbon dioxide from solution eliminated the difference in response to forskolin. Carbonic anhydrase is involved in cAMP activated bicarbonate secretion in canine airway epithelium (85). PPAR γ regulation of carbonic anhydrase effect on forskolin response in NHBE cells requires further study.

Another implication of reduced bicarbonate influx is the potential effect on the reduction in baseline current observed in pioglitazone-treated cells. Reduced intracellular bicarbonate reduces cAMP generation by soluble adenylylase (13, 88, 93). As

mentioned in Chapter 1, cAMP activates anion secretion and the reduction in bicarbonate influx may reduce basal anion secretion by decreasing the amount of basal flux through cAMP-sensitive channels like CFTR.

An interesting observation was made when activators of purinergic receptors were used. P1 receptors and select P2 receptors act through activation of adenylate cyclase, the same mechanism as forskolin. However, no difference in SCC was seen in activation with NECA on either membrane and ATP applied apically increased SCC compared to controls. While the apical ATP SCC response is complex and involves Ca^{2+} -dependent signaling, NECA responses are strictly due to increasing cytosolic cAMP. This result is contradictory to the forskolin response observed in PPAR γ agonist treated monolayers. This observed difference in effect may be due to several different factors. The first possibility is that the NECA response is due to a local increase in cAMP and only local factors are affected. As forskolin activates adenylate cyclase in the whole cell, additional proteins could be activated. A change in expression of forskolin response-activated proteins would result in the difference observed between forskolin and NECA. Another possibility is that the expression of adenylate cyclase has been reduced by PPAR γ treatment. The association of related proteins in the plasma membrane would explain why the NECA responses are not affected, but when the entire cell cAMP levels are increased the difference is evident. Based on the NECA and apical ATP responses, alteration in forskolin response-activated proteins appears to be the most likely cause of the difference in SCC response to cAMP. However, further research is needed to determine the cause of this difference.

To ensure that PPAR γ effects on forskolin stimulated anion secretion were not a product of changes in CFTR expression, several experimental methods were employed. Apical membrane studies were used to evaluate anion flux through CFTR. The use of bdKHS on the basolateral membrane electrically removed the basolateral membrane and created an apical to basolateral chloride gradient. In this preparation, there was no statistical difference between PPAR γ agonist treated and control cells. However, in the majority of trials, PPAR γ agonist treatment increased the apical conductance. To confirm this result, western blot and RT-qPCR experiments were used. Western blot showed no change in CFTR expression, while PCR data showed an increase in CFTR mRNA. Promoter studies were used to determine the genetic effect of PPAR γ on CFTR transcription. Both pioglitazone and FMOC-L-leucine increased promoter activity of CFTR reporter plasmids in A549 cells. The western blot data shows no change in CFTR expression after PPAR γ agonist treatment, promoter activity and PCR data show an increase in CFTR transcription, and the functional results are not clear. While CFTR transcription is increased by PPAR γ in NHBE cells, it is uncertain if PPAR γ causes changes in the expression of CFTR protein. It appears that NHBE cells have a cellular mechanism to regulate the quantity of CFTR translated and expressed on the cell membrane.

The responses to carbachol in control and PPAR γ agonist treated cells provide clues to genetic regulation of the calcium-dependent signaling pathway. PPAR γ agonist treatment reduced the second-phase of the carbachol response. This reduction is hypothesized to result from a decrease in intracellular calcium. Experiments in lcKHS also demonstrated that this difference is due to decreased Cl $^-$ secretion by PPAR γ treated

monolayers. This result was confirmed in bfKHS as the difference between control and pioglitazone-treated monolayers remained. The effects of PPAR γ on the Ca²⁺-signaling pathway appear to be post-receptor as all ion transport modulators that induced anion secretion due to increases in cytosolic Ca²⁺ showed reduced SCC in PPAR γ agonist treated cells. However, research suggests that the ATP response is due to IP3 receptor activation airway epithelial cells and the decreased calcium responses in NHBE monolayers is likely due to PPAR γ regulatory effects on this signaling pathway (74). This decreased anion secretion is not due to changes in calcium-sensitive potassium conductance, as inhibitor experiments showed that K⁺ channel activity is not affected by PPAR γ activation with pioglitazone, and apical conductance was not affected as mentioned above. The reduction in calcium-activated SCC may be a result of impaired calcium release from the ER.

The response to apical ATP is complex and may involve two separate Ca²⁺ dependent signaling pathways. P2Y are receptors expressed on the apical membrane of NHBE cells and it is possible that P2X receptors are also apically expressed. P2X receptors function as plasma membrane calcium channels, while P2Y receptors are metabotropic. In pioglitazone-treated cells, an increase in the first-phase response suggests that P2X receptors may be involved, due to the speed of the anion secretion increase. A reduction in the second-phase response suggests a decrease in P2Y receptor associated Ca²⁺-dependent SCC. The reduction in second-phase response is consistent with observations of other calcium-signaling agonists. The changes in apical ATP response are import for clinical applications of PPAR γ agonist therapy as ATP-analogues

are in clinical use for treatment of airway disease (7, 41, 54). Further research into the effects of PPAR γ on ATP induced anion secretion.

4.3 Future Applications of PPAR γ Agonists in Airway Disease Therapy

Beyond the current uses of PPAR γ agonists in metabolic disease therapy, this class of drugs shows potential as a therapy in airway disease. The increase in apical ATP response after PPAR γ treatment has several important implications for therapy. First, if the increase in SCC due to apical ATP is due to activation of CaCC and other alternate chloride channels, PPAR γ may prove to be valuable in the treatment of Cystic Fibrosis. ATP analogues have already been used in CF to increase fluid secretion (7), ATP analogues in conjunction with PPAR γ agonists may increase the therapeutic benefit. This decreased second-phase of the ATP response is particularly important for evaluating the use for CF therapy. Apical ATP responses are increased in CF, due to increased intracellular calcium (55). This increased intracellular calcium response is due to receptor independent changes in calcium handling (61). If PPAR γ agonist-treatment reduces the rise in intracellular calcium, it would cause exacerbations in patient symptoms – especially since the responses observed appear to be receptor independent. The use of ATP analogues has also been suggested for the therapy of COPD, as both an activator of P2Y₂ receptors and due to its ability to inhibit NF- κ B (22, 68). Since CFTR is active in COPD, the increased apical ATP response would increase the therapeutic benefit of PPAR γ agonists in COPD.

In addition, PPAR γ may be useful as an adjunct to physical therapy. Increased shear stress have been shown to increase ATP release from the apical membrane of

airway epithelial cells (32). Increased shear stress as a result of cough or physical therapy could increase ATP release. PPAR γ activation could increase the effectiveness of physical therapies through increased ATP induced anion secretion. High frequency chest wall oscillation (HFCWO) therapy has been shown to increase mucous clearance alone (57), if HFCWO therapy causes increased apical ATP release, PPAR γ agonists may increase the effectiveness of this therapy in airway disease. Unfortunately, if P2 receptor activated Ca²⁺-dependent signaling is affected by PPAR γ agonists, PPAR γ treatment could cause exacerbation of symptoms in CF patients. A possible contraindication of PPAR γ agonists in CF therapy is the report of increased upper airway infections caused by pioglitazone used for treatment of metabolic disorders (33). However, before PPAR γ agonists can be used as adjunct therapy, more research into the effects of PPAR γ on apical ATP response is required.

Due to the nature of PPAR γ control of ion transport, it is likely that more than one protein in the calcium-dependent signaling pathway has been affected. The alterations in this signaling pathway may provide clues to the effects of PPAR γ in other tissues. PPAR γ regulation is specific to individual promoters and cell types; therefore, cell types that express PPAR γ regulated genes may be affected in a similar manner to NHBE cells. In addition to the regulation of specific cellular proteins, changes in the Ca²⁺ signaling pathway will also alter the function of cells exposed to PPAR γ agonists. Calcium-dependent signaling is important in inflammation, insulin release, and airway smooth muscle contraction (3, 65, 68, 77, 79, 91). While consistent reduction in SCC in response to agonists that elevate intracellular calcium was observed in NHBE cells exposed to PPAR γ agonists, no direct measurements of intracellular calcium were performed.

Downregulation of calcium-dependent signaling may prove advantageous in specific disease states.

PPAR γ agonist therapy may also be advantageous in treatment of obstructive airway disease. In addition to the anti-inflammatory and ion transport effects of PPAR γ in the lungs, PPAR γ agonist treatment may affect bronchoconstriction. Epithelium-dependent bronchodilation is increased by apical ATP activated chloride secretion (27). The increased apical ATP SCC response after PPAR γ agonist treatment may increase epithelium-dependent bronchodilation. The decrease in calcium-dependent signaling may also improve asthma symptoms. Muscarinic receptors also mediate bronchoconstriction via calcium-dependent signaling in airway smooth muscle (6). Downregulation of the calcium-dependent signaling pathway would ameliorate bronchoconstriction in asthma due to muscarinic activation. Anti-muscarinic therapy has also been suggested as a therapy for COPD (6). If the effects of PPAR γ on smooth muscle parallel that of the bronchial epithelium, the bronchoconstriction due to other molecules may also be interrupted. However, the effects of PPAR γ on calcium signaling in airway smooth muscle have not been observed and will require research into the effects of PPAR γ agonists on airway smooth muscle. In addition to potential effects on bronchoconstriction, PPAR γ agonists have been shown to exert a more favorable anti-inflammatory effect on airway smooth muscle (56). Ciglitazone also inhibits asthma-associated airway remodeling in the mouse model (35, 44). Asthmatic airways also show increased expression of CaCC-1 (36), and the apparent reduction in Ca²⁺ signaling after PPAR γ activation could reduce secretion through CaCC.

Chapter 5 Conclusions

PPAR γ represents a novel method of transcriptional control of ion secretion in the lung. While PPAR γ is normally associated with regulation of metabolism, in this thesis it was observed to regulate multiple genes involved in lung epithelial ion transport. PPAR γ is a genetic regulator of ion transport in human bronchial epithelia – a function that is accomplished through regulation of ion transport proteins and signaling pathways. PPAR γ agonists regulate both the cAMP and Ca²⁺ signaling pathways activated by a variety of agonists. In addition, it was demonstrated that PPAR γ regulates the expression of NBC1 and CFTR.

Forskolin and carbachol responses were examined in detail to determine ionic contributions to the changes in SCC after PPAR γ agonist treatment. The reduction in forskolin response was observed to be due to decreased bicarbonate secretion, while the decrease in carbachol SCC was due to chloride secretion. Bicarbonate secretion due to forskolin has been previously observed in the Calu-3 model of submucosal gland cells (21). The reduction in NBC1 expression may partially explain the reduction in forskolin response, but the contribution of carbonic anhydrase was not evaluated.

The effects of PPAR γ on expression and function were also examined. PPAR γ increased CFTR promoter activity in reporter assays and increased CFTR mRNA detected by PCR. However, functional and western blot data showed that CFTR expression was not increased. This suggests that while CFTR is regulated by PPAR γ , homeostatic mechanisms prevent the overexpression of CFTR in NHBE cells.

Purine receptor agonists displayed interesting SCC responses in PPAR γ treated cells. NECA responses were not affected at either the apical or basolateral membranes. ATP responses were modulated at both membranes by PPAR γ agonists. Apical ATP SCC responses were increased, while basolateral ATP responses were decreased after PPAR γ agonist treatment. This difference appears to be the result of increased apical calcium-dependent secretion for the apical membrane response and decreased calcium dependent secretion for the basolateral ATP response. Further research is needed to determine the molecular effects of PPAR γ on P2 receptor activated SCC in NHBE cells.

When the response of all intracellular calcium-activating agonists are examined, it can be concluded that the changes in SCC is most likely due to PPAR γ effects on the Ca²⁺ signaling pathway in NHBE cells. Basolateral carbachol, basolateral ATP, and bilateral thapsigargin responses all show reduced SCC. The apical ATP response also showed a decreased second-phase response possibly implicating decreased intracellular calcium. However, the complexity of the calcium-dependent signaling pathway makes identification of potential targets difficult.

The increased response to apical ATP suggests a potential role of PPAR γ agonists as adjunct therapy in lung disease. ATP analogues are currently in clinical use for airway diseases characterized by decreased mucociliary clearance (7, 54). However, if intracellular calcium is decreased, CaCC channels will be less active in PPAR γ treated cells, contraindicating the usefulness of PPAR γ agonists in cystic fibrosis patients. PPAR γ agonists may prove to be useful in asthma and COPD therapy due to their anti-inflammatory, anti-muscarinic, and pro-ATP response properties. Additionally, the apparent reduction in anion secretion may not be deleterious in mild airway disease

phenotypes. Corticosteroids are commonly used in asthma and COPD and these drugs increase sodium absorption in airway epithelia (60). The net effect of increasing sodium absorption is the same as reducing anion secretion – reduction in ASL volume. Clinical studies could demonstrate whether the reduction in anion secretion is detrimental in patients with inflammatory airway disease.

There are several important considerations when examining the research presented in this thesis. First, the expression of cell surface receptors was not examined. While the decreases in Ca^{2+} signaling occurred regardless of agonist, the expression of cell surface receptors may explain some of the differences observed. Second, PPAR γ agonists may prove useful for the treatment of airway disease. Finally, the Ussing chamber preparation is independent of local modulators released by smooth muscle, local nerves, and other cells. While NHBE cells appear to absorb sodium in their basal state, they may have a secretory function with the combined effect of local modulators occurs in vivo. Both muscarinic and β -adrenergic receptors are present on lung epithelial cells and may contribute to the net ion transport observed in these cells in vivo (66, 91). The research presented in this thesis shows that PPAR γ is a regulator of ion transport in NHBE cells. Endogenous PPAR γ agonists are local modulators that regulate the ion transport of NHBE cells in both basal and disease states. Exogenous PPAR γ agonists may provide an effective therapy in a wide variety of airway diseases.

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